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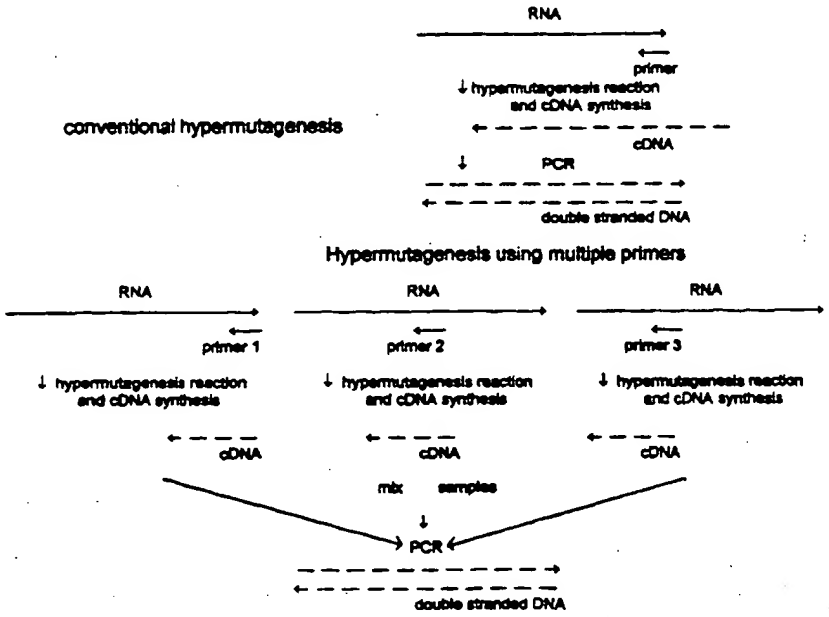

(57) Abstract

The present invention features a method for introducing hypermutations into a target DNA or RNA sequence of interest, characterized in that said method comprises the steps of: (a) transcribing a RNA into DNA in a reaction mixture comprising a reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and (b) recovering said DNA sequences.

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HYPERMUTAGENESIS

1. Field of the Invention

The present invention relates to a method for introducing in vitro multiple mutations into a DNA or RNA sequence of interest or a target DNA sequence hereinafter called the technique of hypermutagenesis. More specifically, the present invention relates to a method for producing a collection of hypermutated genomic DNA, cDNA and RNA sequences in a simple one step reaction. The present invention further relates to the use of the method of the present invention to develop novel polypeptides, peptides or improved genetic expression systems for biotechnical applications. The present invention also relates to antibodies produced against the mutated polypeptides and to the use of said polypeptides in diagnosis and vaccination fields or in gene therapy or for therapeutic purposes. The method of the present invention can also be used for identification of functionally important regions in proteins and gene regulatory regions.

2. State of the Art

Proteins and peptides play an essential role in all biochemical and many pharmaceutical applications. The primary structure of a polypeptide, as encoded by its respective gene, contains the determinants for its folding to a specific three-dimensional structure and also for all of its properties. Therefore, by changing the base sequence of the respective gene, all properties of a protein can be in principle modified towards a desired optimum. Gradual accumulation of

random base substitution mutations is an essential mechanism that creates genetic diversity, which serves as material for genetic selection under various conditions during the development of novel proteins for technical applications. The essential features of such methods include the random and efficient generation of base substitutions and the optimization of the ratio between the twelve different types of base substitutions.

Until a few years ago, only two methods were available to directly study the relationship between the structure and function of a protein. These methods included the chemical modification of the side chains of amino acids that form the primary sequence of the protein and X-ray diffraction of protein crystals. There were, however, problems associated with these two methods which problems included the fact that the proteins had to be available in large quantity and be of high purity.

A variety of different methods have been recently developed for studying ther relationship between the structure and function of a protein by introducing mutations into cloned genes and screening for specific properties of the proteins generated by the mutations. All of these methods involve the use of enzymes and chemicals which cleave, degrade or synthesize DNA. For example, the previously described random in vitro mutagenesis methods include the use of mutagenic chemicals (Myers et al Science, 229 pp, 242-249 (1985)), oligonucleotide mutagenesis of heteroduplex or cassette type (Hutchinson et al., PNAS, USA, 83, pp710-714 (1986), enzymatic misincorporation (Shortle and Lin, Genetics 110, pp539-555 (1985) and the enzymatic random mutagenesis method described by

Lehtovaara et al., Protein Engineering, vol. 2, no.1 pp 63-68 (1988).

The problems encountered with the above-mentionned methods are multifold. First of all, many of the above-described methods are laborious, result in a incomplete libraries, are expensive or are not efficient enough to permit complex screening systems for randomly mutagenized genes and their protein products. For example, in oligonucleotide-mediated mutagenesis, the larger and more complex the mutation, the lower the efficiency with which it will be generated. It is known that when large deletions are generated approximately 50-fold lower efficiency occurs than in mutations involving only local changes in sequences.

Moreover, the frequencies of mutations of the closest prior art methods are on the order of 10^{-3} to 10^{-4} per base which is quite low. The number of mutated clones resulting from these procedures is at best about 60% or less, while the number of mutations per clone is usually less than or equal to 2.

For example, EP 0 285 123 describes a method of generating one or two point mutations in each mutatnt and generating a library containing all possible single base mutations of a gene by a process that involves limited elongation of a primer, enzymatic misincorporation using Klenow polymerase to generate point mutations into the molecular population and completion of the mutagenized molecules in forms that can be amplified and further isolated by molecular cloning. The frequency of the mutants generated by this method is about 60% with each mutant containing on the average 1.8 base substitutions.

Pjura et al in Protein Science, 2, pp. 2217-2225 (1993) describe a misincorporation mutagenesis method using a single-stranded phage M13mp18 and avian myeloma virus reverse transcriptase to identify thermostable mutants of T4 lysozyme. However, one of the problems with this assay was that mutations occured outside the intended area of the M13 genome targeted by the initial primer-extension reaction; i.e., in the lac operator gene. In addition, the overall mutation rate remained low.

Therefore, there is a need in this art to generate a method to mutate cDNA sequences that is efficient, practical and overcomes the problems associated with the known prior art methods.

RNA viruses replicate with an intrinsic error some 300 times greater than DNA based microbes and approximately 10^6 times greater than eukaryotic genomes. See, Drake, PNAS, USA 90 pp4171-4175 (1993). This is a consequence of total lack of replication proof reading machinery and results in an intrinsic nucleotide substitution error of about 0.05 to 1 per genome per cycle. See, Holland et al., Curr. Top. Microbiol. Immunol., 176, pp.1-20 (1992). Occasionally there is a total breakdown in replication fidelity giving rise to hypermutated genomes encoding hundreds of monotonously substituted bases. See, for example Vartanian et al PNAS, USA, 91 pp. 3092-3096 (1994).

To date there are two different types of known hypermutated RNA viral genomes. Adenosine (A)-->Inosine (I) hypermutation of measles and vesicular stomatitis viral genomes which are thought to result from post-transcriptional enzymatic modification of adenosine to inosine. Bass et al., Cell, 56, 331 (1989). G-->A hypermutated genomes have been described for a large number of lentiviruses including human

immunodeficiency virus type 1 (HIV-1) and were hypothesized to arise during reverse transcriptase as a result of monotonous substitution of dCTP by dTTP due to the localized depletion of intracellular dCTP (deoxycytidine triphosphates). Vartanian et al, J. Virol. 65, pp. 1779-1788 (1991); Vartanian et al (supra).

The present invention started to evolve from the fact that hypermutated sequences naturally occur during reverse transcription in HIV-1 and were possibly due to the depletion of intracellular dCTP. See, for example, the Abstract by Martinez et al, "Hypermutagenesis of RNA using HIV-1 Reverse Transcriptase and Biased dNTP Concentrations", Retroviruses, May 25, 1994.

Generally, the method of the present invention involves transcribing a RNA into a DNA using any reverse transcriptase, including those derived from a lentivirus or a hepadnavirus, for example, and using various biased concentrations of deoxynucleoside triphosphates to produce hypermutations. The DNA is recovered and may be PCR amplified, cloned into an expression vector, expressed in a suitable host microorganism and screened for appropriate traits that may have been generated by the hypermutation.

These and other objects are achieved by the present invention as evidenced by the object and summary of the invention, the description of the preferred embodiments and the claims as indicated below.

OBJECTS AND SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method to produce a collection

of hypermutated cDNA, genomic DNA and RNA sequences much more efficiently and in greater quantity and with a greater mutation frequency than those presently described in the prior art.

Yet another object of the present invention is to provide a method to produce a collection of hypermutated cDNA, genomic DNA and RNA sequences that can be produced in a simple one or two step reaction and to introduce mutations into cDNA, genomic DNA and RNA.

Yet another object of the present invention is to provide novel polypeptides and improved genetic expression systems by using the method of the present invention.

Yet another object of the present invention is to produce monotonous nucleic acid substitutions in the DNA sequence of interest of G --> A or U/T --> C or A --> G or C --> T.

It is yet another object of the present invention to produce mixed mutations of the DNA sequence of interest involving the nucleic acid substitutions of G --> A and U/T --> C or A --> G and C --> T.

In another aspect, the present invention relates to the use of a reverse transcriptase such as a lentiviral or a hepadnaviral reverse transcriptase or a retroviral reverse transcriptase in the present process capable of introducing a frequency of mutation in a target nucleotide sequence between 10^{-1} and 10^{-3}, to transcribe <u>in vitro</u> RNA into a DNA.

The present invention features a method for introducing hypermutations into a target DNA or RNA sequence of interest, characterized in that said method comprises the steps of:

(a) transcribing a RNA into DNA in a reaction mixture comprising a reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(b) recovering said DNA sequences.

In a preferred embodiment the present invention features a method for introducing hypermutations into a target DNA or RNA sequence, characterized in that said method comprises the steps of :

(a) cloning said target DNA sequence into a vector that is capable of regenerating RNA in vitro;

(b) transcribing said RNA into DNA in a reaction mixture comprising a reverse transcriptase, preferably a lentivirus reverse transcriptase or a hepadnavirus reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(c) recovering said DNA sequences.

In yet another preferred embodiment the present invention features a method for introducing hypermutations into a DNA or RNA sequence of interest, characterized in that said method comprises the steps of :

(a) amplifying said target DNA or RNA using oligonucloetide primers encoding promoter sequences for T7 and T3 RNA polymerases to produce RNA derived from the amplified products;

(b) transcribing said RNA into DNA in a reaction mixture comprising a reverse trancriptase, preferably a lentivirus reverse transcriptase or a hepadnavirus reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations

and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(c) recovering said DNA sequences.

Brief Description of the Drawings

Fig. 1 is Southern Blot illustrating the efficiency of cDNA synthesis with varying concentrations of deoxynucleoside triphosphates.

Fig. 2 illustrates the nucleic acid sequences of cDNA produced by the method of the present invention at varying deoxynucleoside triphosphate concentrations.

Fig. 3 illustrates the forward and backward primer sequences utilized in the method of the present invention for the R67 DHFR gene.

Fig. 4 illustrates the forward and backward primer sequences containing T3 and T7 promoter sequences for PCR amplification.

Fig. 5 is the DNA and amino acid sequences of a R67 gene that can be hypermutated by the methods in the present invention.

Fig. 6 depicts a modification of the invention in which multiple primers are used to increase the efficiency of cDNA synthesis during the hypermutagenesis reaction.

Fig. 7 depicts the use of multiple primers according to the technique of Fig. 6 to hypermutate the R67 gene. Modified R67 DHFR gene sequence with respect to that given in Brisson and Hohn, Gene 28, 271-275, 1984 is shown in Fig. 7. Amino acid sequence in the

three letter code is given above the nucleic acid sequence.

Fig. 8 is a diagram of another modification of the invention using dialysis to increase the efficiency of cDNA synthesis during the hypermutation reaction.

Fig. 9 depicts the frequency distribution of G-->A transitions per clone generated by reverse transcription with HIV-1 reverse transcriptase, avian myeloblastosis virus (AMV), reverse transcriptase and Moloney murine leukemia virus (MoMLV) reverse transcriptase.

Fig. 10 illustrates a bar graph obtained after sequencing a total of 202 and 190 clones using the HIV-1 reverse transcriptase reactions, which illustrates the distribution of G-->A transitions in various hypermutated sequences. The horizontal line gives the average value per site for the combined data sets.

Detailed Description of the Preferred Embodiments of the Invention

As used herein the term "hypermutated" means that the DNA sequence has at least three base pairs changes that differ from the unmutated DNA sequence.

As used herein, the term "varying concentrations of deoxynucleoside triphosphates" means differing concentrations of dCTP, dTTP, dATP and dGTP.

As used herein, the term "biased pyrimidine concentrations" means the concentration ratio of dCTP to dTTP in the reaction mixture is not equal or is unbalanced. For example, there is a low concentration of dCTP and a high concentration of dTTP.

As used herein, the term "biased purine concentrations means that the concentration ratio of dATP and dGTP in the reaction mixture is not equal or is unbalanced. For example, there is a low concentration of dATP and a high concentration of dGTP.

As used herein, the term "partially complementary", when referring to the oligonucleotide primers means that the oligonucleotide sequence does not have to be 100% complementary to the RNA sequence; i.e., that each nucleotide must correspond to the complementary nucleotide. It is preferable that at least 10 nucleotides are complementary and more preferably from about 15 to 20.

As used herein, the word "unmutated" encompasses, any natural nucleic acid sequence or a genetically engineered or chemically modified nucleic acid sequence, which sequences have not been hypermutated.

The "base pairs" means normal or analogues molecules used in nucleotide sequences.

The term "natural DNA sequence" connotes that the DNA sequence is one that is not altered from that found in nature.

As used herein, the symbol "G-->A", when referring to hypermutations, means that a guanine nucleotide is substituted by an adenine-based nucleotide.

As used herein, the symbol "U-->C" when referring to hypermutations means that a uracil nucleotide is substituted by a cytidine nucleotide.

More specifically, the present invention relates to a process for generating a library of hypermutant DNA sequences which differ from the natural DNA sequences by changes made in the nucleotide base pairs.

Mutant proteins with the desired properties can be identified and expressed in a suitable host. Specific conditions can then be designed to screen the transformants for the particular trait in question.

Also contemplated by the present invention is the use of the process of hypermutagenesis to modify any protein such as enzymes, receptors, restriction enzymes, monoclonal or polyclonal antibodies, nucleic acid polymerases (i.e., reverse transcriptase) and the like to confer on the protein different properties from the natural properties of the protein. For example, the modification by hypermutagenesis of restriction enzyme genes may result in restriction enzymes having novel restriction sites. A monoclonal antibody can be altered by hypermutagenesis to improve its affinity. The catalytic site of an enzyme can be hypermutated to improve its catalytic activity or alter its substrate specificity.

Thus, for example, modified subtilisin by altering two amino acids to produce subtiligase has recently been used for the total synthesis of a variant of ribonuclease A containing nonnatural catalytic residues. See, Science, 226, 243 (1994). Such modified proteins can be produced by the process of hypermutagenesis of the present invention.

Also contemplated by the present invention are antibodies produced against the mutated polypeptides and to the use of said polypeptides for diagnosis and vaccination fields or gene therapy or therapeutics.

Novel forms of antibiotics are also contemplated by the present invention and can be obtained by hypermutating the genes involved in the biosynthesis of presently known antibiotics.

Other uses of the novel proteins produced by the method of the present invention are also contemplated but are too numerous to mention.

Generally, the present invention involves the production of a hypermutated DNA sequence by starting with, for example, isolating mRNA from total RNA of the sequence of a target DNA sequence or fragment of interest.

By target DNA sequence or DNA sequence of interest is meant any DNA sequence that is or will be known in the art that can be subjected to the hypermutagenesis process such as cDNA and genomic DNA. RNA can be subjected to hypermutation, also. The choice of the target DNA or RNA sequence depends, of course, on the type of mutant one wants to create and the type of trait one wants to instill on the protein.

The various types of DNA or RNA sequences include, but are not limited to, DNA sequences of enzymes, restriction enzymes, nucleic acid polymerases, monoclonal or polyclonal antibodies, genes involved in the synthesis of antibiotics, gene transfer vector sequences and the like as well as fragments of these sequences. It is well within the knowledge of a person skilled in the art to choose the target DNA sequences.

Total RNA can be isolated by a variety of methods known in the art, such as proteinase K digestion, followed by phenol:chloroform extraction, guanidinium thiocyanate extraction, followed by cesium chloride gradients, guanidine hydrochloride and organic solvent extraction, and the like.

mRNA can then be isolated from total RNA by methods known in the art. These methods include the use of oligo(dT)-cellulose, poly(U) Sepharose, adsorption

to and elution from poly(U) filters or nitrocellulose membrane filters, and the like. It is preferable to use oligo(dT) cellulose chromatography in isolating mRNA following the procedure described by Sambrook et al, Molecular Cloning, A Laboratory Manual (1989).

Alternatively, a DNA sequence of interest or a fragment thereof can be purified by methods known in the art and cloned into a plasmid or phage vector that is capable of generating RNA in vitro that is complementary to either of the two strands of foreign DNA inserted into the polycloning site. Preferred vectors of the present invention include pGEM-3, pGEM-4, pGEM-3Z, pGEM-3Zf(-), Bluescript M13$^-$, Bluescript M13$^+$, pBluescript SK$^-$, Bluescript KS$^+$ and variations thereof. It is preferable to use a vector that has a promoter on each side of the polycloning site; these promoters include the T7 promoter, the SP6 promoter, the T3 promoter and the like.

The RNA of interest can be generated by using the insert DNA as a template for in vitro transcription using a RNA polymerase. Any RNA polymerase can, in principle, be used such as RNA polymerase I, RNA polymerase II, RNA polymerase III, T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase and the like provided that the corresponding promoter sequences flank the target sequence. It is preferable to use T7, T3 or SP6 RNA polymerase.

The reaction conditions for generating RNA in a plasmid may vary according to the vector system and DNA insert. These reaction conditions are well known in the art and can be adjusted accordingly to optimize the production of RNA. One preferred example of the reaction conditions is: 500 μM each of deoxynucleoside triphosphates, 100 ng of template, 0.3 U/μl of RNase

inhibitor and 2U/μl of RNA polymerase nad buffer in a final volume of 100 μl.

After the RNA is produced, it is then further purified by phenol extraction and ethanol precipitation according to Sambrook et al (supra).

In another preferred embodiment of the present invention, the target DNA can be PCR amplified using oligonucleotide primers encoding the promoter sequences for the T7, SP6 and T3 RNA polymerases. Thus, RNA can be derived directly from the amplified products, without resorting to an intermediate cloning step. An example of these T7 and T3 RNA polymerase primers for use in this embodiment are exemplified in Figure 4.

Thus, RNA can be obtained by any method, said RNA corresponding to the target DNA sequence of interest.

After acquiring the RNA or mRNA by the various methods set forth above, the RNA or mRNA is then reverse transcribed into DNA by using a reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutation and an oligonucleotide primer under conditions allowing the favorable transcription of RNA into DNA in a reaction mixture.

Any reverse transcriptases can be utilized in the present invention. Such reverse transcriptase can be isolatable from a retrovirus capable of infecting eukaryotic cells. For example, these reverse transcriptases may include human immunodeficiency virus Type 1 (HIV-1) reverse transcriptase, human immunodeficiency virus Type 2 (HIV-2) reverse transcriptase, simian immunodeficiency virus reverse transcriptase, feline immunodeficiency virus reverse transcriptase reverse transcriptase derived from the

ungulate lentiviruses, and lentivirus reverse transcriptases that are hypermutated variations of the lentivirus reverse transcriptase. It is preferable to use HIV-1 reverse transcriptase or HIV-2 reverse transcriptase in the transcription method of the present invention.

The invention concerns itself particularly with lentiviral reverse transcriptases. However, it is known that the reverse transcriptases of avian myeloblastosis virus (Amv) and Moloney murine leukemia virus (MoMLV) are capable of hypermutagenizing RNA also. However, in the current protocol they are not capable of the same degree of mutation. Nonetheless they, as well as reverse transcriptases derived from other retroviruses, are potentially useful reagents and can be utilized in the present method.

Reverse transcriptases also associated with the hepadnaviruses (e.g., Human hepatitis B virus (HBV), Woodchuck hepatitis virus (WHV), Ground squirrel hepatitis virus (GSHV), Duck hepatitis B virus (DHBV) and Heron hepatitis B virus (HHBV) or with caulimoviruses (e.g., cauliflower mosaic virus (CaMV)) can also be utilized. Thus, the above-described reverse transcriptases from these viruses are potentially useful reagents and can be utilized in the present method.

The amount of reverse transcriptase utilized in the reaction mixture of the present invention should be in a large molar excess in respect to the RNA.

The ratio of molar excess of reverse transcriptase to RNA may vary from about 30:1 to about 50:1 (reverse transcriptase: RNA). It is preferable to use about a 50:1 molar excess.

For example, if 0.5 pmol of RNA template is utilized, the reverse transcriptase is present in the reaction mixture on the order of between 15.0 pmol to about 25.0 pmol.

An oligonucleotide primer that is partially complementary to the 3' end the target RNA is selected on the basis of the corresponding target RNA sequence and is utilized in the reaction mixture for the transcription reaction. This oligonucleotide primer can vary in length from about 15 to about 50 nucleotides. The primer should match with about 10 to 15 nucleotides of the complementary target sequence. These oligonucleotide primers can be prepared by methods known in the art and disclosed by Sambrook et al (supra).

In another preferred embodiment of the present invention, multiple primers can also be utilized during the hypermutagenesis reaction, as set forth in Figure 6. Thus, at least three primers can be utilized, preferably from 3 to 6 and most preferably from 2 to 4. The number of primers depends on the length of the target RNA to be hypermutated. Thus, the longer the target RNA, the more primers can be utilized.

The concentration of the oligonucleotide primer should be in excess of the target RNA concentration. However, usually about 2 pmol is utilized in the reaction mixture as described below.

Varying concentrations of deoxynucleoside triphosphates are then added in the transcription reaction mixture. The concentration and type of deoxynucleoside triphosphates may vary depending on the type of hypermutation in the DNA sequence of interest one wants to obtain. For example, if one wants to obtain a G-->A hypermutation, then a low concentration

of dCTP substrate is used compared to dTTP which is used in a high concentration. By altering the dATP/dGTP ratios, i.e., using a low concentration of dATP compared to dGTP which is used in a high concentration, hypermutation of U-->C can be seen. dITP can also be substituted for dGTP in the reaction mixture and when used in high concentrations as compared to a low concentration dATP hypermutations of U-->C occur. These above-described hypermutations are "monotonous hypermutations". It is also possible to obtain a mixture of G-->A and U-->C hypermutations by using a low ratio of dCTP/dTTP and low ratio of dATP/dGTP nucleotide triphosphates during cDNA synthesis.

The molar concentrations of the specific deoxynucleoside triphosphates will of course depend upon the type of hypermutation selected. Therefore, a very low dCTP/dTTP ratio of about 10^{-4} will produce extensive hypermutation while, in contrast to a hundred fold less ratio of about 10^{-2} will produce less hypermutations. For instance, if a low degree of hypermutations (< 5%) is desired, the deoxynucleoside triphosphate bias should not be too low; i.e., dCTP/dTTP should be in a ratio of about 1/5000. However, if the production of a greater degree of hypermutations is desired, then, for example, the dCTP/dTTP ratio can vary between 1/15000 to 1/50000. The amount hypermutation in the target cDNA sequence can therefore be controlled by altering the ratios of dCTP/dTTP, dATP/dITP and dATP/dGTP. Therefore, one can select the concentrations of the deoxynucleoside triphosphates according to the amount of hypermutations one wants in the cDNA sequence.

In a most preferred embodiment of the invention, the concentrations of deoxynucleoside triphosphates may vary from 1 nM up to 440 μM in a transcription reaction

mixture. Moreover, one can generate hypermutations in a DNA or RNA of about 100% (or total target nucleotide replacement of a DNA or RNA sequence) by the method of the present invention. Therefore, if the target DNA or RNA sequence contains, for example, 200 bp nucleotides, all the target nucleotides can be altered. However, it is possible to alter the DNA or RNA sequence to a lesser extent, also which can range from about 2% to about 99%. More preferably from about 2% to about 20%. More specifically, for a G-->A hypermutation, it is most preferable to use 1 to 100 nM of dCTP, about 440 μM of dTTP, about 40 μM of dATP, and about 20 μM of dGTP or dITP in the transcription reaction mixture. In contrast, for a type of hypermutation from U-->C, it is most preferred to use approximately 10 μM dCTP, 44 μM of dTTP, 3 to 100 nM of dATP and 200 μM of dGTP in the transcription reaction mixture. For a mixed type of hypermutation, that is G-->A plus U-->C, it is most preferable to use about 1 to 100 nM of dCTP, about 440 μM of dTTP, 1 to 100 nM of dATP, and about 200 μM of dGTP or dITP in the transcription reaction mixture.

It must be realized that there is considerable latitude in some of the dNTP concentrations. Thus in order to accomplish G-->A hypermutation the key element is that the dCTP concentration must be low (1-100 nM) and the dTTP must be high (> 100 μM). The concentration of dATP (40 μM) and dGTP (20 μM) were chosen as they approximate to the mean intracellular concentration in mammalian lymphocytes. In this example, the only important feature of the dATP and dGTP concentrations is that they are saturating (> 1-5 μM). Thus any concentration beyond this is satisfactory.

The reverse transcriptase reaction is generally run in a reaction buffer that also can vary, according to the procedures of Sambrook et al supra. However, it

is most preferable to use a buffer consisting of 50 mM Hepes (pH 7), 15 mM Mg aspartate, 10 mM DTT, 55 mM K acetate, and 15 mM NaCl or 50 mM Hepes (pH8), 15 mM MG Aspartate, 10 mM DTT, 130 mM KAcetate and 15 mM Nacl.

In a preferred embodiment of the present invention the reaction mixture for the transcription reaction consists of 0.5 pmol template RNA, 2 pmol oligonucleotide primer, in 50 μl reaction buffer, 0.3 U/ml of RNase inhibitor and 15 pmol (6.25 units) of HIV-1 reverse transcriptase. However, the person skilled in the art can alter this reaction mixture accordingly and obtain the target cDNA according to methods known in the art.

The reverse transcriptase reaction can be over a period of time that varies of course with the length of the DNA insert and the oligonucleotide primer utilized. For example, when annealing the oligonucleotide primer 1 minute incubation at various temperatures and with various subtitutes, is generally performed prior to the addition of the reverse transcriptase. After the addition of the reverse transcriptase, the reaction is usually run at about 37°C for about 3 hours. More specifically, the primers can be annealed to the template DNA by first heating to about 65°C for 1 minute, followed by incubation at 37°C for 1 minute. After which the reverse transcriptase was added.

These reaction conditions can be adjusted and optimized accordingly to obtain optimal DNA or RNA production.

After the DNA or RNA has been generated, the DNA or RNA is recovered by any method known in the art, such as those decribed by Sambrook et al, supra or in European Patent Application No. 518 313. More preferably, the DNA or RNA can be amplified by PCR with

forward and backward primers to generate sufficient DNA to further clone into a plasmid for regenerating. One can also use the T7 and/or T3 promoter sequences in the PCR primers. This way, the target DNA or RNA sequence of interest can be cycled in the PCR process. For example, a protocol for one PCR cycle would require 1) PCR amplification of target sequence, 2) DNase treatment, 3) RNA synthesis using the T7 or T3 dependant RNA polymerases, 4) target DNA or RNA synthesis using, for example, the lentivirus reverse transcriptase and biased dNTP pools.

Once hypermutated, and the target DNA or RNA sequences have been sufficiently recovered, they can be further ligated into any expression vector and transformed in a host microorganism. Any expression vector known in the art can be utilized, and any host microorganism can be utilized. Examples of these systems are described by Sambrook et al (supra) or in PCT No. WO-93 19785.

Once expressed, the hypermutated DNA or RNA sequences may be screened. There are a large number of screening systems known in the art such as genetic complementation in bacterial yeast and mammalian systems and the like. The screening system will of course depend on the particular gene of study.

For example, R67 DHFR is a dehydrogenase which specifically reduces dihydrofolate to tetrahydrofolate. If hypermutated, R67 DHFR DNA can be ligated into a expression vector such as pTrc99A and used to transform strains of E. coli deficient in one of many dihydrogenases. It might be possible to then select for a mutant of broadened or altered specificity.

In yet another preferred embodiment of the present invention, the hypermutagenesis method is repeated at

least two times, preferably between 2 and 20 times, most preferably between 2 and 50 times, utilizing different oligonucleotide primers for each reaction. Pools of different hypermutated cDNA are formed and then combined. The combined pool can then be further hypermutated using the method of the present invention or the cDNA can be joined from each pool by homologous recombination.

Yet another preferred embodiment of the present invention involves hypermutating RNA sequences based on biased deoxyribonucleoside triphosphate (dNTP) concentrations using dialysis. Instead of carrying out the elongation reaction mixture containing the RNA primers and buffers, a test tube containing a nitrocellulose filter is utilized. The reaction buffer and dNTPs are placed in a Petri dish which dialize into the filter starting the hypermutagenesis reaction.

The mean frequency of G-->A transitions (G-->A) per data set may be calculated as the number of transitions/(number of clones sequences x number of G residues in the sequence). The frequency of G-->A transitions clearly varies as a function of concentration of dCTP, being for small values of f, $f/(1 - f) = [dTTP]/[dCTP] \sum \alpha i/$number of clones sequenced x number of G residues in the sequence where $\alpha i = [(Vmax/km)dTTP]/[(Vmax/km)dCTP]i$ and i = number of clones sequenced. See, for example, Fersht., A. (1983), Enzyme Structure and Mechanism, 2nd Edition, W.H. Freeman and Company, U.S.A., p. 98-120.

A hypermutated protein or peptide characterized by a mutation frequency between 10^{-1} to 10^{-3} as compared to the target molecule is also contemplated by the present invention. Purified monoclonal antibodies made from the hypermutated DNA sequences and immunogenetic molecules

made from the process of the present invention are also contemplated.

Chimeric proteins containing mutations at least about 20%, preferably about 5% to 50% and 80%, preferably between 95 to 5% unmutated sequence is also contemplated by the present invention.

The present invention also concerns the preparation of cell lines containing the hypermutated genes carried by a vector and stably integrated into the genome.

This invention is also applicable to plant cells, plant cell cultures and nucleic acid sequences encoding plant proteins.

As discussed above, the applications of hypermutagenesis are too numerous to mention. However, useful applications of the hypermutagenesis process contemplated by the present invention are the following :

1) The modification of restriction endonuclease genes with a view to isolating restriction enzymes with novel restriction sites.

2) The modification of monoclonal antibody VH and VL genes with a view to improving the affinity of the monoclonal antibody. The affinity of monoclonal antibodies are notoriously low. Any improvement in their affinity would increase the specificity and the diminution in the quantity of monoclonal used per sample/reagent/kit.

3) Modification of substrate specificity. The hypermutagenesis of a gene conferring the metabolism of some toxic product or hydrocarbon, new activities are sought by plating the library of hypermutated genes on

plates containing the toxic product or hydrocarbon. The uses of such novel products in pollution control is considerable.

4) Enhanced catalytic activity. Hypermutagenesis can speed up the search for variant genes products with improved nitrogen fixing activities, photosynthetic properties, protease activity, etc..

5) Producing novel DNA-dependent DNA polymerases (e.g., E. coli PolI, the Klenow fragment, Taq polymerases and other thermostable enzymes), DNA-dependent RNA polymerases (e.g., bacteriophage T7, T3, SP6 enzymes) and DNA dependent DNA polymerases (e.g., reverse transcriptions from any retrovirus, Tth and TetZ thermostable enzymes, Klenow fragment (under certain conditions), that when hypermutated may yield novel variants capable of hypermutagenezing RNA/DNA more efficiently than previously described.

In the specific case of reverse transcriptases, central to the hypermutagenesis method, judicious screening following hypermutagenesis of the HIV-1 reverse transcriptase gene, or a fragment thereof, it might be possible to select for variants of reduced fidelity. Alternatively, screening for variants with an enhanced ability to elongate beyond a mismatch would be practically most useful. This is because cDNA synthesis in the presence of biased dNTP pools is rather inefficient. However, if elongation could be improved, this would enable us to go to even more biased dNTP ratios (currently 44,000 fold bias) which may be extended to beyond 400,000 fold. This would enable us to increase the average degree of hypermutation by a factor of 2 or more.

If, instead of using hypermutagenesis of the HIV-1 reverse transcriptase gene, the Taq polymerase gene was

studied then, following judicious screening, it can be possible to find variants of Taq with a poorer fidelity. In fact, hypermutagenesis of any of the above-mentioned polymerases might lead to a crop of variants with altered properties, i.e., increased heat stability, reduced fidelity, enhanced elongation beyond a mismatch, smaller size, more protease resistant of solvant insensitive or combinations thereof.

6) Yet another application is the hypermutagenesis of the envelope protein of retroviral vectors altering their cell tropism. Currently many of the retroviral vectors used use envelope proteins of a wide tropism, i.e., they infect a wide variety of different cell types. The gene corresponding to the envelope protein, or parts thereof, is hypermutated and variants can be obtained with a more restricted tropism. Such reagents can be of use in gene therapy in that it would enable a more specific targetting of the desired gene.

7) Yet another application is the hypermutagenesis of DNA/RNA sequences derived from plants or other microorganisms.

The experimental approach to the above-mentioned applications to be exploited would follow very much the experimental protocol outlined in the present application for the hypermutagenesis of target DNA or RNA sequences or fragments of a polymerase gene.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given, it being understood that the same are intended only as illustrative and in no wise limitative.

EXAMPLE 1

DNA from M13mp18 recombinant containing the env V1-V2 regions of HIV-1 B40 (described by Vartanian et al, J. Virol., 65, pp. 1779-1788 (1991)) was PCR-amplified with oligonucleotide 1 (5' GCGAAGCTT AATTTAATACGACTCACTATAGGGACAAAGCCTAAAGCCATGTGTA) and oligonucleotide 2 (5' GCGGAATTCTAATGTATGGGAATTGGCTCAA). Oligonucleotide 1 contains the T7 RNA polymerase promoter sequence (underlined) allowing production of plus strand transcripts of the env VA-V2 region. The 342bp DNA fragment, with unique HindIII and EcoRI restriction sites (bold face), was digested and ligated into a pBluescript SK$^+$ vector. The resulting plasmid was digested with HindIII and EcoRI, and the fragment was purified from a 2% agarose gel.

Insert DNA was used as template for in vitro transcription using T7 RNA polymerase. Reaction conditions were: 40 mM Tris.HCl (pH 8), 30 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml RNase/DNase-free BSA, 500 μM each NTP, 100 ng of template, 0.3 U/μl of RNase inhibitor (Pharmacia) and 2 U/μl of T7 RNA polymerase (Pharmacia) in a final volume of 100 μl. After incubation for 1 hour at 37°C, the DNA template was digested with 0.075 U/μl RNase-free DNase I (Pharmacia) for 30 min at 37°C. RNA was phenol extracted and ethanol precipitated.

The reverse transcription reaction buffer was 50 mM Hepes (pH 7), 15 mM MgAspartate, 10 mM DTT, 55 mM KAcetate, 15 mM NaCl, and varying dNTP concentrations (see legend to Fig. 1). Two picomoles of oligonucleotide 3 (5' GCGTCTAGAAGTATCATTATCTATTGGTA, complementary to positions 224 to 253 of the 342bp plus strand DNA fragment), was annealed to 0.5 pmol of the template RNA in 50 μl of the reaction by first heating to 65°C for 1 min. followed by incubation at 37°C for 1

min. after which 0.3 U/μl of RNase inhibitor and 15 pmol (6.25 units) of HIV-1 RTase (Boehringer) were added. The reaction was incubated at 37°C for 3 hours.

In order to recover sufficient material for subsequent cloning, cDNA was amplified by PCR with oligonucleotide 3 and oligonucleotide 4 (5' GCGGTCGACCAAAGCCTAAAGCCA) producing a 231 bp DNA fragment with restriction sites at its end, XbaI and SalI respectively (bold face). Optimized PCR conditions were: 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl (pH 8.3), 200 μM of each dNTP, 20 μl of the reverse transcription reaction and 2.5 U of Taq DNA polymerase (Cetus) in a final volume of 100μl. Annealing, extension and denaturation cycling parameters were: 37°C (30s), 72°C (30s) and 95°C (30s) for 2 cycles and 55°C (30s), 72°C (30s) and 95°C (30s) for 10 cycles. Southern blot analysis was made on 5% of the PCR amplified material electrophoresed through an agarose gel, and blotted into a nylon membrane by capillary transfer. The filter was hybridized with a ^{32}P 5'-labelled probe 5' TGTAAAA(^{T}/C)(^{T}/C)AACCCCAC(^{T}/C)C, complementary to positins 53 to 72 of the env V1-V2 342 bp minus strand. The specific 231 bp PCR fragments were purified from a 2% agarose gel, digested with XbaI and SalI and ligated into pBluescript SK^+ vector. Clones were dideoxy-sequenced using the Taq dye primer cycle sequencing kit (Applied Biosystems), with resolution of the products on a Applied Biosystems 373A sequencer. 334 recombinants were sequenced and some of the mutants sequences are shown in Figure 2. The frequency of mutations are described in Table 1.

dNTP (μM)				Plaques analyzed			G-->A subs	fG-->A*	Deletions Insertions	Other point mutations
C	T	A	G	Total	WT	Mutated				
10	44	40	20	47#	46	1	0	0	1 0	0#
1	440	40	20	90#	78	12	12	4.1×10^{-3}	0 0	1#
0.1	440	40	20	31	10	21	38	3.8×10^{-2}	3 0	10
0.03	440	40	20	27	2	25	70	8.1×10^{-2}	3 4	4
0.01	440	40	20	38	6	32	146	1.2×10^{-1}	3 2	10
							U-->C subs	fU-->C*		
10	44	0.1	200	18	16	2	2	2.5×10^{-3}	0 0	0
10	44	0.03	200	33	6	27	58	4.0×10^{-2}	3 0	8
							G-->A U-->C	fG-->A fU-->C		
0.03	440	0.03	200	27	3	24	40 24	4.6×10^{-2} 2.0x10-2	9 1	9
0.03	0.03	0.03	0.03	22	19	3	1 0	1.5×10^{-3}	0 0	2

Table 1

Example 2

A dihyrofolate reductase (DHFR) gene encoded by an E. coli plasmid R67 and will hereafter be referred to as R67 DHFR or simply R67 utilized as the target DNA sequence of interest. The corresponding coding sequences were amplified by PCR from a plasmid clone under standard conditions (2.5 mM $MgCl_2$, 50 mM Tris-HCl pH 8.3, 50 mM KCl, 200 μM each dNTP, 100 ng plasmid DNA, 100 pmol of each primer (see Figure 3), 2.5 U Taq polymerase (Cetus); 15 cycles with the following cycling parameters; 30 sec 92°C, 30 sec 55°C and 30 sec 72°C). The resulting product was digested with SacI and BamHI restriction endonucleases and cloned into the BlueScript vector pSK+. Recombinant clones were checked by sequencing.

RNA was derived from the cloned R67 gene sequences by using the T7 RNA polymerase in an in vitro reaction. Before the T7 reaction, supercoiled plasmid DNA was linearized with a one cut restriction endonuclease (e.g., SacI) which cleaves 3' to the insert. Reaction conditions for the T7 in vitro reaction were: 40 mM Tris.HCl (pH 8), 30 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml RNase/ DNase-free bovine serum albumin (BSA), 500 μM each NTP, 100 ng of template, 0.3 U/μl of RNase inhibitor (Pharmacia) and 2 U/μl of T7 RNA polymerase (Pharmacia) in a final volume of 100 μl.

After incubation for 1 hour at 37°C, the DNA template was digested with 0.075 U/μl RNAse-free DNase I (Pharmacia) for 30 min at 37°C in order to eliminate template DNA. RNA was phenol extracted and ethanol precipitated.

For hypermutagenesis the reverse transcription reaction buffer was 50 mM Hepes (pH 7), 15 mM

MgAspartate, 10 mM DTT, 55 mM KAcetate, 15 mM NaCl. The deoxynucleoside triphosphate (dNTP) concentrations were varied according to the permutations shown in the Table below :

Type of permutation	dCTP	dTTP	dATP	dGTP
G-->A	1-100 nM	440 μM	40 μM	20 μM
U-->C	10 μM	44 μM	1-100 nM	200 μM
G-->A + U-->C	1-100 nM	440 μM	1-100 nM	200 μM

Two picomoles of oligonucleotide (backward primer (Fig. 3), complementary to positions just 3' to the target sequence to be hypermutated), was annealed to 0.5 pmol of the template RNA in 50 μl of the reaction by first heating to 65°C for 1 min. followed by incubation at 37°C for 1 min. after which 0.3 U/μl of RNase inhibitor and 15 pmol (6.25 units) of HIV-1 RTase (Boehringer) were added. The reaction was incubated at 37°C for 3 hours.

In order to recover sufficient material for subsequent cloning, cDNA was amplified by PCR with the forward and backward primer pair (Fig. 3). Optimized PCR conditions were: 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 5-20 μl of the reverse transcription reaction and 2.5 U of Taq DNA polymerase (Cetus) in a final volume of 100 μl. Annealing, extension and denaturation cycling parameters were: 37°C (30s), 72°C (30s) and 95°C (30s) for 2 cycles and 55°C (30s), 72°C(30s) and 95°C (30s) for 10 cycles. The low annealing temperature used in the first two cycles was designed to favourize amplification of RNA sequences containing mutations in the PCR primer target sequence.

Once amplified, the DNA was cleaved by BamHI and SacI restriction endonucleases which cut within the primer

sequences (Fig. 3). The products were then ligated into the pTrc99A expression vector (Stratagene) at an equimolar ratio of insert to vector. After transformation of competent E. coli cells and plated out on to standard LB (Lauria broth) plates supplemented with 100μg/ml trimethoprim (TMP) and 100 μg/ml ampicilin (AMP).

To identify the nature of the hypermutated R67 genes, TMP resistant colonies were grown up singly, plasmid DNA extracted and the DNA was sequenced. The wild type R67 sequence is shown in Fig. 5.

EXAMPLE 3

In this example, RNA corresponding to the "palm" domain of nucleic acid polymerases (about 600 base pairs), is being subject to hypermutagenesis. The palm domain encodes the catalytic residues responsible for nucleic acid polymerization. The precise example here is the gene encoding the Klenow fragment of E. coli DNA polymerase I.

A plasmid "cassette" is made carrying the Klenow fragment gene under the control of a suitable promoter and including convenient and unique restriction sites around the region to be hypermutagenized. This cassette vector is obtained by conventional and existing mutagenesis procedures such as those described by Sambrook et al (supra).

The "palm" domain of the Klenow fragment is first amplified from the complete Klenow fragment using the standard PCR method; 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each dNTP, 100 mg plasmid DNA, 100 pmol of each primer in Fig. 3 and 2.5 U Taq polymerase (Cetus), in 15 cycles with the following cycling parameters : 30 sec 92°C, 30 sec 55°C and 30 sec 72°C. The amplification primers have T7 and T3 promoter sequences at their 5' expremities, i.e.,

forward primer,
5' clamp - restriction site - ATTAACCCTCACTAAAGGGA (T3 promoter) - target specific sequence
reverse primer
5' clamp - restriction site - AATTTAATACGACTCACTATAGGG (T7 promoter) - target specific sequence.

Following amplification RNA is made from either strand in a standard in vitro reaction. Reaction conditions for the T7 in vitro reaction were: 40 mM Tris.HCl (pH 8), 30 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml RNase/ DNase-free bovine serum albumin (BSA), 500 μM each NTP, 100 ng of template, 0.3 U/μl of RNase inhibitor (Pharmacia) and 2 U/μl of T7 RNA polymerase (Pharmacia) in a final volume of 100 μl.

After incubation for 1 hour at 37°C, the DNA template was digested with 0.075 U/μl RNAse-free DNase I (Pharmacia) for 30 min at 37°C in order to eliminate template DNA. RNA was phenol extracted and ethanol precipitated.

For hypermutagenesis the reverse transcription reaction buffer was 50 mM Hepes (pH 7), 15 mM MgAspartate, 10 mM DTT, 55 mM KAcetate, 15 mM NaCl. The deoxynucleoside triphosphate (dNTP) concentrations were varied according to the permutations shown in the Table below :

Type of permutation	dCTP	dTTP	dATP	dGTP
G-->A	1-100 nM	440 μM	40 μM	20 μM
U-->C	10 μM	44 μM	1-100 nM	200 μM
G-->A + U-->C	1-100 nM	440 μM	1-100 nM	200 μM

Two picomoles of oligonucleotide (backward primer (Fig. 3), complementary to positions just 3' to the target sequence to be hypermutated), was annealed to 0.5 pmol of

the template RNA in 50 µl of the reaction by first heating to 65°C for 1 min. followed by incubation at 37°C for 1 min. after which 0.3 U/µl of RNase inhibitor and 15 pmol (6.25 units) of HIV-1 RTase (Boehringer) were added. The reaction was incubated at 37°C for 3 hours.

In order to recover sufficient material for subsequent cloning, cDNA is amplified by PCR with the forward and backward primer pair (Fig. 3). Optimized PCR conditions are 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl (pH 8.3), 200 µM of each dNTP, 5-20 µl of the reverse transcription reaction and 2.5 U of Taq DNA polymerase (Cetus) in a final volume of 100 µl. Annealing, extension and denaturation cycling parameters would be : 37°C (30s), 72°C (30s) and 95°C(30s) for 2 cycles and 55°C (30s), 72°C (30s) and 95°C (30s) for 10 cycles. The low annealing temperature used in the first two cycles is designedto favorize amplification of RNA sequences containing mutations in the PCR primer target sequence.

Once amplified the material is cleaved by the restriction sites introduced into the target sequence which gave rise to the cassette vector. The products are ligated into the cassette vector. Genetic or phenotypic screening, known by the man skilled in the art (Maniatis, Sambrook) is then employed.

EXAMPLE 4

Following Example 1 set forth above, the following reverse transcriptases of a retrovirus infecting mammalian cells and capable of inducing a mutation frequency comprised between 10^{-1} and 10^{-3} are utilized in lieu of HIV-1 reverse transcriptase : HIV-2 reverse transcriptase; feline immunodeficiency virus reverse transcriptase, an ungulate antivirus reverse transcriptase, simian immunodeficiency virus reverse transcriptase, avian

myeloblastis reverse transcriptase, moloney murine leukemia virus reverse transcriptase, th hepadnavirus reverse transcriptase and caulimovirus reverse transcriptase, and hypermutated variants.

EXAMPLE 5

Cell-lines containing a hypermutated gene carried by a vector or integrated in their genome also are prepared.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the scope thereof. Accordingly, it is intended that the scope of the present invention be limited by the scope of the following claims, including equivalents thereof.

EXAMPLE 6

A feature of the hypermutagenesis reaction is that cDNA systhesis is not too efficient, being inversely proportional to the ratio of the concentrations of dCTP and dTTP, i.e., [dCTP]/[dTTP]. Given the power of PCR, it is usually possible to recover sufficient material to clone. However, it represents an impediment to exaggerating the [dCTP]/[dTTP] ratio, which would increase the degree of substitution of G by A. In order to alleviate these problems, this invention provides two ways of increasing the efficiency of cDNA synthesis during the hypermutagenesis reaction.

The first is to use multiple primers during the hypermutagenesis reaction instead of one (see, Fig. 6). Conceptually, it is very simple. A precise example is furnished in Fig. 7, where the use of the R67 encoded dihydrofolate reductase (DHFR) gene as target sequence is

exemplified. Of course, the hypermutagenesis reaction can be utilized with any RNA sequence. More particularly, instead of adding a single oligodesoxyribonucleotide (or primer) to the RNA, three reactions are set up with only one primer in each (primers 1, 2 and 3 in Figs. 6 and 7). With very biased [dCTP]/[dTTP] ratios, short segments, less than the full length complementary sequence, can be produced.

After the hypermutagenesis reactions, a sample from each of the three reactions is taken and mixed together in PCR buffer along with the outer pair of primers 3 and 4 (Figs. 6 and 7). The cDNA fragments made in the hypermutagenesis reaction serve as the initial templates for PCR using the left primer 4. PCR recombination (Meyerhans et al. 1991) assures assembly of the full length, yet hypermutated, R67 sequences. Once PCR material is obtained, conventional molecular biology is employed.

While this example mentions a biased [dCTP]/[dTTP] ratio, it will be understood that this technique can equally be performed with a biased [dATP]/[dGTP] ratio, as previously mentioned. In the present example, three primers were used to hypermutate R67 RNA. Three have been used efficiently. However, nothing prevents the use of 2, 4, or more primers. Indeed, the longer the target RNA, then the more the number of primers necessary.

Thus, this technique makes it possible to recover hypermutated RNA more easily and with greater efficiency than before. This embodiment of the invention resides in the recognition that multiple primers allow recovery of short cDNA fragments made during the reaction. It relies on the recombinatorial power of PCR to assemble DNA fragments. See, Meyerhans, A., Vartanian, J.P. & Wain-Hobson, S. (1992) Nucl. Acids Res. **20**, 521-523 and Stemmer, W.P.C. (1994), Nature **370**, 389-391.

EXAMPLE 7

A second modification of the invention involves a conceptually very different physical means to hypermutagenize RNA. This modification is shown in Fig. 8 and involves hypermutagenesis by dialysis. This embodiment stems from the realization that during cDNA synthesis, under biased deoxyribonucleoside triphosphate (dNTP) concentrations, the dCTP concentration, for example, is not constant but is being depleted resulting in poor cDNA synthesis. This poses the problem as to how a given bias can be maintained. Following is a simple solution, which allows cDNA synthesis under highly biased but constant dNTP ratios. Once again, the R67 DHFR gene RNA is used as an example, but of course any other RNA sequence can be used.

The components are those previously described, i.e., R67 RNA, the outer primer 3 (Fig. 7), reaction buffer, HIV-1 reverse transcriptase, but no dNTPs. The composition of this mix was: 50 mM Hepes (pH 7), 15 mM Mg Aspartate, 10 mM DTT, 55 mM K acetate, 15 mM NaCl. Two picomoles of oligonucleotide (primer 3, Fig. 6), complementary to positions just 3' to the target sequence to be hypermutated, were annealed to 0.5 pmol of the template RNA in 50 μl of the reaction by first heating to 65°C for 1 min. followed by incubation at 37°C for 1 min. after which 0.3 U/μl of RNase inhibitor and 15 pmol (6.25 units) of HIV-1 RTase (Boehringer) were added. Instead of carrying out the reaction in a closed plastic tube, the 50 μl (typically, but not exclusively) reaction volume is placed upon a 0.025 μm nitrocellulose filter as in Fig. 8.

In the meantime, approximately 10-15 ml of reaction buffer and dNTPs are placed in a Petri dish and prewarmed at 37°C without shaking for approximately 20-30 minutes. The composition of this mix was: 50 mM Hepes (pH 7), 15 mM Mg Aspartate, 10 mM DTT, 55 mM K acetate, 15 mM NaCl. The

deoxynucleoside triphosphate (dNTP) concentrations are varied. Typical permutations are shown below:

Type of permutation	dCTP	dTTP	dATP	dGTP
G-->A	3-100 nM	440 μM	40 μM	20 μM
U-->C	10 μM	44 μM	3-100 nM	200 μM
G-->A + U-->C	3-100 nM	440 μM	3-100 nM	200 μM

The reaction volume was placed on the cellulose-base filter and was left at 37°C for 3 hours without shaking. The dNTPs dialyze into the 50 μl reaction volume in a matter of minutes thereby starting the hypermutagenesis reaction. However, given the huge excess of dNTPs in the volume under the filter (some 200 fold), the dNTP ratios hardly change throughout the reaction. After hypermutagenesis, a sample was amplified by PCR using oligos 3 and 4 (Fig. 7) by standard techniques.

EXAMPLE 8

A M13mp18 clone containing the env V1-V2 regions of HIV-1 B40, Vartanian, J.P. et al, J. Virology, 65, pp. 1779-1788 (1991), was PCR-amplified with oligonucleotide 1 (5' **GCGAAGCTT**CAAAGCCTAAAGCCATGTGTA) and oligonucleotide 2 (5' GCG**GAATTC**TAATGTATGGGAATTGGCTCAA). The 317 bp DNA fragment, with unique HindIII and EcoRI restriction sites (shown in bold), was digested and ligated into a pBluescript SK⁺ vector. One microgram of the resulting plasmid was digested with EcoRI, and used as a substrate for in vitro transcription using T7 RNA polymerase as described by Martinez et al, Proc. Natl. Acad. Sci., USA, 91, pp. 11787-11791 (1994). RNA was phenol extracted and ethanol precipitated. The reverse transcription reaction buffer was 50 mM Hepes (pH 7 or pH 8, as shown in Table I below), 15 mM Mg Aspartate, 10 mM DTT, 130 mM KAcetate, 15 mM NaCl, and varying dNTP

concentrations, as shown in Table II below). Two picomoles of oligonucleotide 3 (5' GCGTCTAGAAGTATCATTATCTATTGGTA, complementary to positions 199 to 228 of the 317 bp plus strand DNA fragment, was annealed to 0.5 pmol of the template RNA in 50 µl of the reaction by first heating to 65°C for 1 min. followed by incubation at 37°C for 1 min., after which 15 pmol (6.25 units) of either the HIV-1 RTase (Boehringer), AMV RTase (Promega) or MoMLV RTase (Gibco) and RNase inhibitor (0.3 U/µl final, Pharmacia) were added. The reaction was incubated at 37°C for 3 hours. Deoxynucleotide triphosphates were purchased from Pharmacia while dUTP, dITP and ITP were from Boehringer.

In order to recover sufficient material for subsequent cloning, cDNA was amplified by 12 cycles of PCR with oligonucleotide 3 and oligonucleotide 4 (5' GCGGTCGACCAAAGCCTAAAGCCA) producing a 231 bp DNA fragment with restriction sites at its end, XbaI and SalI respectively (shown in bold, Martinez (1994) supra). The specific 231 bp PCR fragments were purified from a 2 % low melting point agarose gel, digested with XbaI and SalI and ligated into M13mp18 RF DNA and transformed into E. coli XL-1 blue. Clones were sequenced by the standard dideoxy method using M13 universal primer.

The results are shown in Table II below. All reactions were run at pH 7.0 except these indicated in Table II as pH 8.

TABLE II

dNTP Bias	Reverse Transcriptase	dNTPs/μM				Substitutions	
		T	C	G	A	G→A	$\bar{f}_{G\to A}$
T>>C	HIV-1	440	0.1	20	40	47	3.8 x10^{-2}
		440	0.03	20	40	483	7.9 x10^{-2}
		440	0.01	20	40	866	1.3 x10^{-1}
		440	0.003	20	40	63	1.7 x10^{-1}
T>>C	AMV	440	0.1	20	40	8	1.2 x10^{-2}
		440	0.03	20	40	60	4.6 x10^{-2}
		440	0.01	20	40	79	9.4 x10^{-2}
T>>C	MoMLV	440	0.1	20	40	5	8.2 x10^{-3}
		440	0.03	20	40	12	9.8 x10^{-3}
		440	0.01	20	40	17	2.1 x10^{-2}
		U	C	G	A	G→A	$\bar{f}_{G\to A}$
U>>C	HIV-1	440	0.1	20	40	4	1.1 x10^{-2}
		440	0.03	20	40	10	1.6 x10^{-2}
		440	0.01	20	40	12	3.1 x10^{-2}
		440	0.003	20	40	6	3.9 x10^{-2}
		T	C	G	A	C→A	$\bar{f}_{C\to A}$
T>>G	HIV-1	440	10	0.01	40	5	9 x10^{-3}
		440	10	0.003	40	2	8 x10^{-3}
		T	C	G	A	C→T	$\bar{f}_{C\to T}$
A>>G	HIV-1	44	10	0.03	400	1	1.5 x10^{-3}
		T	C	G	A	A→G	$\bar{f}_{A\to G}$
C>>T	HIV-1	0.01	100	20	40	4	5.2 x10^{-3}
		T	C	I	A	U→C	$\bar{f}_{U\to C}$
I>>A	HIV-1	44	10	200	0.03	0	<2 x10^{-3}
Inosine in RNA		T	C	G	A	G→A	$\bar{f}_{G\to A}$
T>>C	HIV-1	440	0.1	20	40	36	5.2 x10^{-2}
		T	C	G	A	G→A	$\bar{f}_{G\to A}$
T>>C	HIV-1 pH8	440	0.1	20	40	6	9 x10^{-3}
		440	0.03	20	40	30	5.5 x10^{-2}
		440	0.01	20	40	78	1.2 x10^{-1}

The frequency distribution of G-->A transitions per clone generated by reverse transcription by either of the three reverse transcriptases is given in Figure 9. For HIV-1, AMV and MoMLV reverse transcriptases, the maximum number of substitutions per clone was respectively 8, 6 and 2 at 30 nM dCTP and 12, 7 and 3 at 10 nM dCTP respectively. The mean number of transitions per clone was 2.5, 1.5 and 0.3 for the 30 nM dCTP HIV-1, AMV and MoMLV RTase reactions and 4.3, 2.8 and 0.7 for the 10 nM dCTP reaction.

The mean frequency of G-->A transitions ($\bar{f}_{G-->A}$) per data set were calculated as the number of transitions/(number of clones sequences x number of G residues in sequence, i.e., 32) and are given in Table 1. The frequency of G-->A transitions ($\bar{f}_{G-->A}$ or simply $\bar{f}$), clearly varies as a function of [dCTP] the relationship, being for small values of $\bar{f}$, $\bar{f}/(1-\bar{f})$ = [dTTP]/[dCTP] $\alpha_i/32$, where $\alpha_i = [(V_{max}/K_m)dTTP]/[(V_{max}/K_m)dCTP]_i$ and i = 32 (see, Fersht, A., supra). In the absence of individual values of α_i for each site they were taken to be equal and constant for each of the 32 G residues, effectively assuming no effect of sequence context (vide infra). The relationship of the pH on the frequency of G-->A hypermutation in fact showed only a very slight difference, statistically insignificant, between fidelity at pH7 and pH8 as a function of dCTP concentration (Table II).

The substitution of dUTP in lieu of dTTP greatly reduced the concentration dependence of G-->A hypermutation by the HIV-1 RTase (Table II). The use of dITP in place of dGTP did not significantly change the mean substitution frequency of the HIV-1 enzyme in the context of U-->C.

EXAMPLE 9

A total of 202 and 190 clones were sequence from the HIV-1 RTase reactions using 10 nM and 30 nM dCTP as set forth in Table 1. The distribution of transitions was normalized to the total number (866 and 483 for the 10 and 30 nM HIV-1 respectively). Figure 10 illustrates the distribution of G-->A transitions in hypermutated sequences. These results illustrate that the sequence context influences the G-->A transitions.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the scope thereof. Accordingly, it is intended that the scope of the present invention is limited by the scope of the following claims, including equivalents thereof.

WHAT IS CLAIMED IS :

1. A method for introducing hypermutations into a target DNA or RNA sequence of interest, characterized in that said method comprises the steps of:

(a) transcribing a RNA into DNA in a reaction mixture comprising a reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(b) recovering said DNA sequences.

2. The method according to Claim 1, wherein said RNA is provided by isolating total RNA from a protein source, which contains the DNA sequence of interest.

3. The method according to Claim 2, wherein mRNA is isolated from said total RNA.

4. The method according to Claim 3, wherein said mRNA is isolated using oligo(dT) cellulose chromatography.

5. The method according to Claim 1, wherein said RNA is provided by using a plasmid or phage vector that generates RNA in vitro when double-stranded DNA is inserted into a polycloning site.

6. The method of Claim 5, wherein said plasmid vector is pGEM-3, pGEM-4, pGEM-3Z, pGEM-3ZF, Bluescript M13$^+$, Bluescript M13$^-$, pBluescript KS$^+$ or pBluescript SK$^+$.

7. The method according to Claim 5 or Claim 6, wherein said vector is pBluescript SK$^+$ or pBluescript KS$^+$.

8. The method according to any one of Claims 1 to 7, wherein said lentivirus reverse transcriptase is selected from the group of human immunodeficiency virus-1 reverse transcriptase, human immunodeficiency virus-2 reverse transcriptase, feline immunodeficiency virus

reverse transcriptase, simian immunodeficiency and hepadnavirus virus reverse transcriptase, ungulate lentivirus reverse transcriptases avian myeloblastosis virus reverse transcriptase, Moloney murine leukemia virus reverse transcriptase and hypermutated variations thereof.

9. The method according to Claim 8, wherein said reverse transcriptase is present in a large molar excess in respect to the RNA.

10. The method according to Claim 9, wherein said reverse transcriptase is present in a ratio of 30:1 or 50:1 in respect to the RNA.

11. The method according to Claim 1, wherein said RNA is transcribed in step (b) at 37°C for 3 hours in said reaction mixture.

12. The method according to any one of Claims 8, 9 and 10, wherein said deoxynucleoside triphosphates are present in the reaction mixture at biased pyrimidine concentrations, more preferably between about 1 nM to 100 nM dCTP and about 440 μM dTTP, about 20 μM dGTP and about 40 μM dATP.

13. The method according to any one of Claims 8, 9 and 10, wherein said deoxynucleoside triphosphates are present in the reaction mixture at biased purine concentrations, more preferably about 1 nM to 100 nM dCTP, about 1 to 100 nM ATP, about 440 μM dTTP and about 200 μM dGTP or dITP.

14. The method according to Claim 12 or 13, wherein said biased pyrimidine concentration produced DNA sequences having G-->A hypermutations and said biased purine concentration produces DNA sequences having U-->C hypermutations.

15. The method according to Claim 1, wherein said d oxynucleoside triphosphates ar present in concentrations to produce a combined G-->A and U-->C hypermutation.

16. The method according to Claim 1, wherein said oligonucleotide primer has about 12 to 50, preferably greater than 10 nucleotides that are complementary to the 3' end of said RNA.

17. A method for introducing hypermutations into a target DNA or RNA sequence, characterized in that said method comprises the steps of :

(a) cloning said target DNA sequence into a vector that is capable of regenerating RNA in vitro;

(b) transcribing said RNA into DNA in a reaction mixture comprising a reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(c) recovering said DNA sequences.

18. The method according to Claim 17, wherein said vector is capable of generating RNA in vitro that is complementary to both strands of the target DNA sequence.

19. The method according to Claim 1 or Claim 17, wherein said hypermutated DNA or RNA sequences are recovered by amplifying said sequences by PCR using oligonucleotide primers.

20. The process according to any one of Claims 1 to 19, wherein said DNA sequences are further ligated into an expression vector and transformed using an appropriate cell host.

21. A process for introducing hypermutations into a target DNA or RNA sequence, characterized in that said process comprises the steps of :

(a) amplifying said target DNA or RNA using oligonucloetide primers encoding promoter sequences for T7 and T3 RNA polymerases to produce RNA derived from the amplified products;

(b) transcribing said RNA into DNA in a reaction mixture comprising a reverse transcriptase, preferably a lentivirus reverse transcriptase, varying biased concentrations of deoxynucleoside triphosphates to produce hypermutations and an oligonucleotide primer that is partially complementary to the 3' end of said RNA; and

(c) recovering said DNA sequences.

22. The method according to any one of Claims 1, 17 or 21, wherein said recovering step (c) involves amplification of the DNA sequence using PCR oligonucleotide primers that are partially complementary to either end of said DNA sequences.

23. The method according to claim 22, which comprises the additional step of cloning into an expression vector the amplified DNA sequences.

24. Use of the method according to any one of Claims 1, 17, 21, 22 or 23, to produce a hypermutated protein.

25. A hypermutated protein or a peptide, characterized by a mutation frequency between 10^{-1} to 10^{-3} compared to the target protein or peptide unmutated sequences.

26. A chimeric hypermutated protein or peptide, characterized by at least 5% of mutated regions compared to 95% of unmutated sequence of the protein or peptide.

27. Purified polyclonal or monoclonal antibodies raised against the products according to Claim 25 or 26.

28. Diagnostic kit for detecting HIV using at least a reagent product as defined in Claim 27.

29. An immunogenic molecule according to Claim 25 or Claim 26.

30. Use as a therapeutical agent of the products claimed in Claims 25, 26, 27 or 29.

31. A hypermutated enzyme for which the gene encoding it comprises a frequency of mutation of 10^{-1} to 10^{-3} mutations compared to the unmutated sequences.

32. Hypermutated nucleic acid sequences, characterized by a frequency of mutation of 10^{-1} to 10^{-3} mutations compared to the unmutated sequences.

33. A reverse transcriptase from any natural, genetically engineered or chemically modified gene sequences encoding a nucleic acid polymerase which is used in a hypermutagenesis reaction.

34. A method for introducing hypermutations into a nucleotide sequence, wherein the method comprises:

(A) providing a reaction mixture consisting essentially of:

(1) a RNA template to be hypermutated;

(2) reverse transcriptase for reverse transcribing the RNA template to form cDNA, wherein the RNA template and reverse transcriptase are in a molar ratio of about 30:1 to about 50:1;

(3) an oligonucleotide primer for initiating cDNA synthesis along the template, wherein the oligonucleotide primer is present in a molar excess relative to the RNA template; and

(4) a mixture of deoxynucleoside triphosphates comprising dATP, dTTP, dGTP, dCTP, wherein molar concentrations of said deoxynucleoside triphosphates are biased such that

(a) the concentration of dCTP is low relative to the concentration of dTTP;

(b) the concentration of dATP is low relative to the concentration of dGTP; or

(c) the sum of the concentrations of dCTP and dATP is low relative to the sum of the concentrations of dTTP and dGTP; and

(B) transcribing said RNA template to form the hypermutated nucleotide sequence having G-->A mutations, u--> C mutations, or mixed G-->A and U-->C mutations in which about 2% to about 99% of the nucleotides in the RNA template are mutated.

35. The method as claimed in claim 34, wherein the reverse transcriptase is HIV-1 reverse transcriptase.

36. The method as claimed in claim 34, wherein the reverse transcriptase is HIV-2 reverse transcriptase.

37. The method as claimed in claim 34, wherein the method is duplicated at least two times, and wherein different oligonucleotide primers are employed each time to thereby form pools of different hypermutated cDNA;

combining the pools; and

joining the different hypermutated cDNA from each pool by homologous recombination.

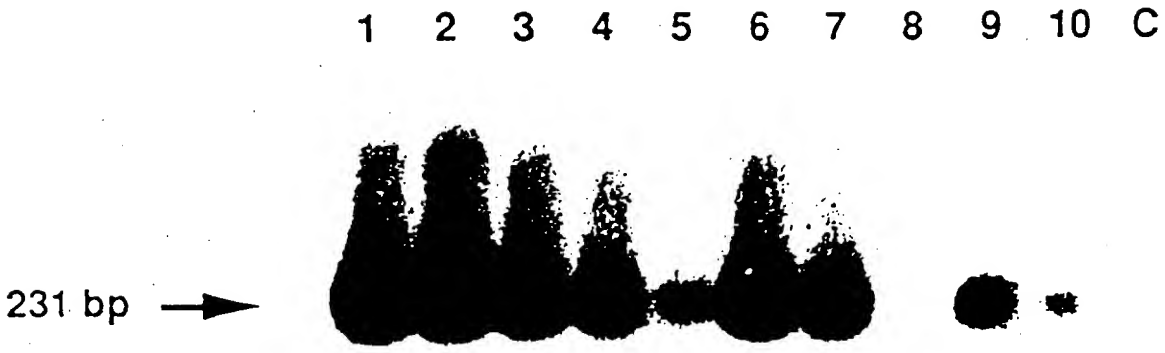
38. The method as cliamed in claim 34, wherein deoxynucleoside triphosphate is in a first reaction zone that is separated by a dialysis membrane from a second reaction zone containing the RNA template, reverse transcriptase, and oligonucleotide primer;

wherein the deoxynucleoside triphosphate enters the second reaction zone by dialysis through said membrane to thereby provide a substantially inuform concentration of deoxynucleosidetriphosphate to the second reaction zone for cDNA synthesis.

39. The method as claimed in claim 34, wherein the ratio of dCTP to dTTP is about 10^{-2} to about 2×10^{-5}.

40. The method as claimed in claim 34, wherein the ratio of dATP to dGTP is about 10^{-2} to about 2×10^{-5}.

41. The method as claimed in claim 34, wherein dATP and dGTP concentration are saturating.



	dCTP	10	10	.10	.03	.01	10	10	10	.03	.03	10
dNTP	dTTP	44	440	440	440	440	44	44	44	440	.03	44
μM	dATP	40	40	40	40	40	.10	.03	.01	.03	.03	40
	dGTP	20	20	20	20	20	200	200	200	200	.03	20

FIGURE 1

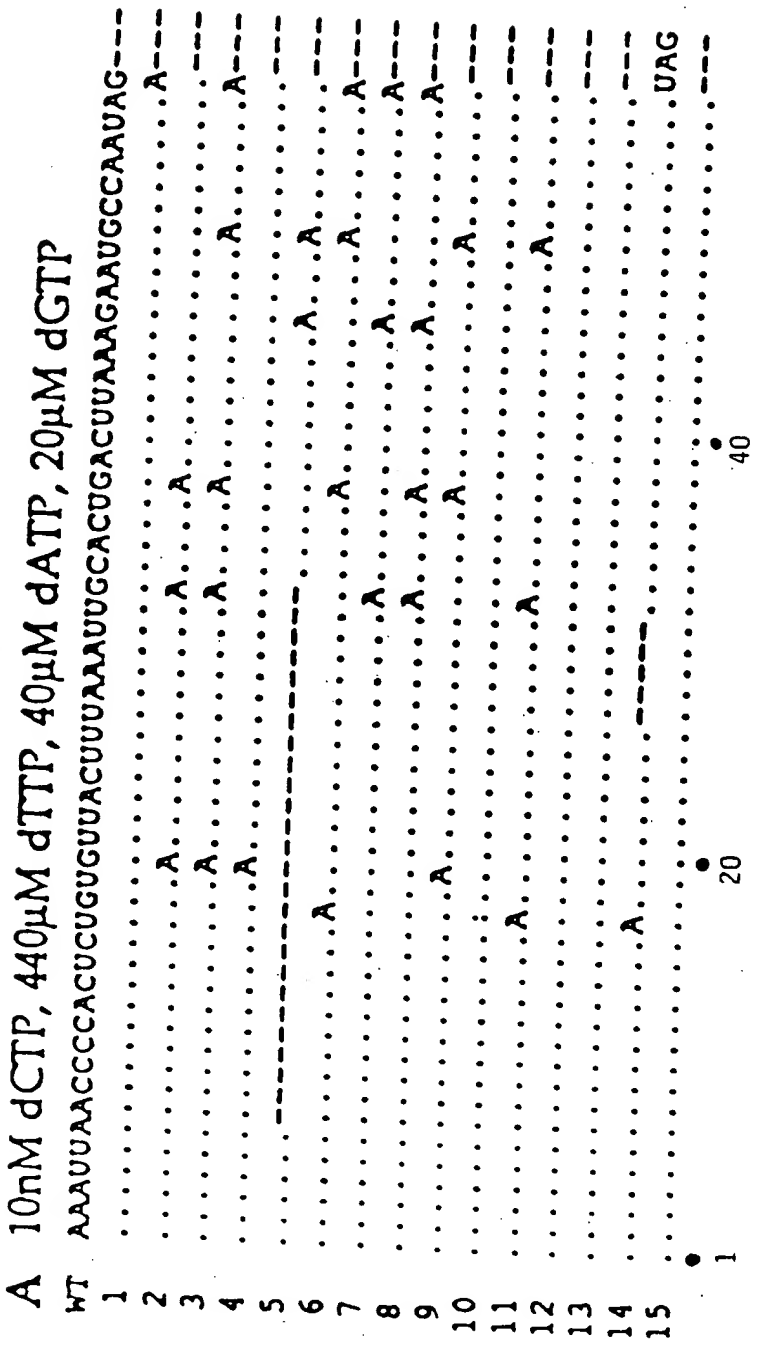

A 10nM dCTP, 440µM dTTP, 40µM dATP, 20µM dGTP
WT AAAUUAACCCCACUCUGUGUUACUUUAAAUUGCACUGACUUAAAGAAUGCCAAUAG---
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
UAG
1
20
40

FIGURE 2

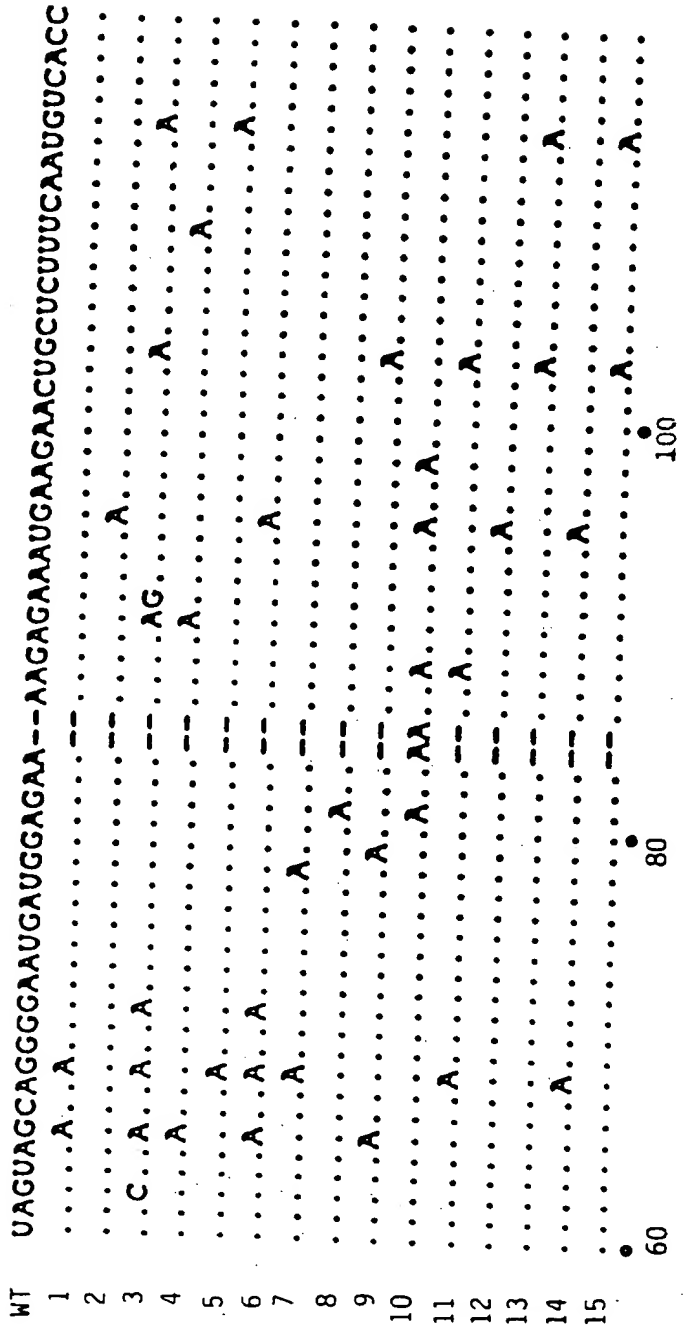


FIGURE 2 (Continued)

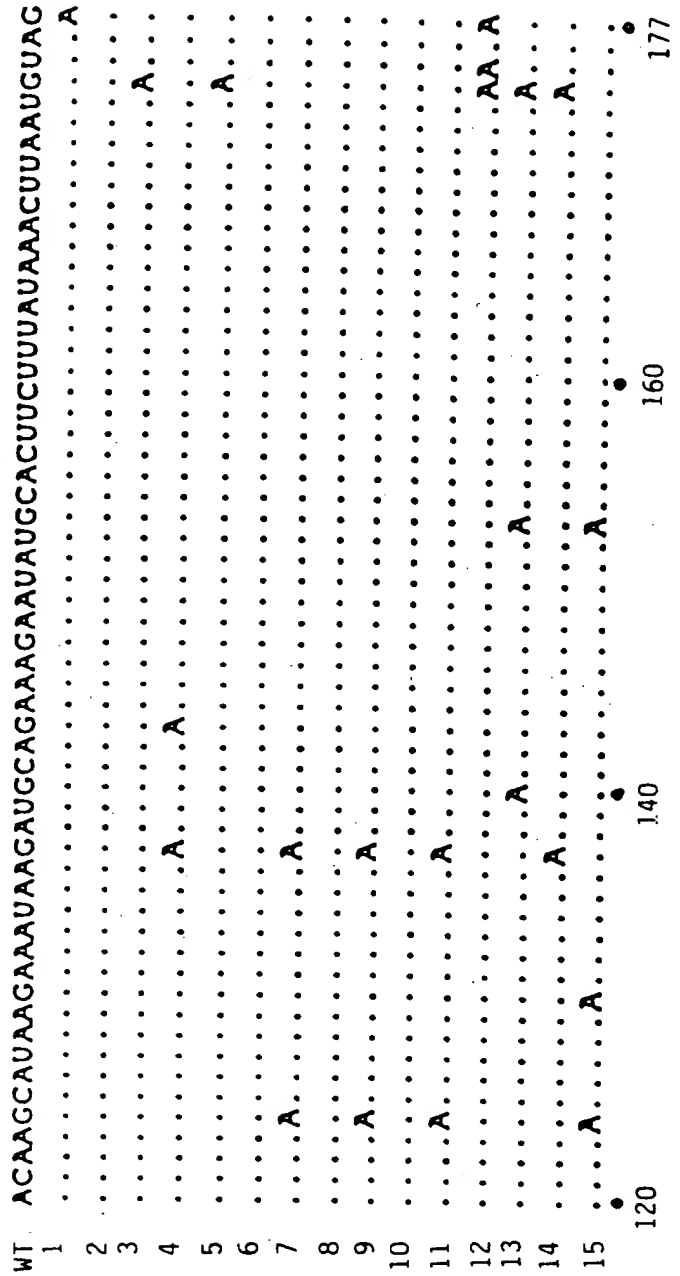


FIGURE 2 (Continued)

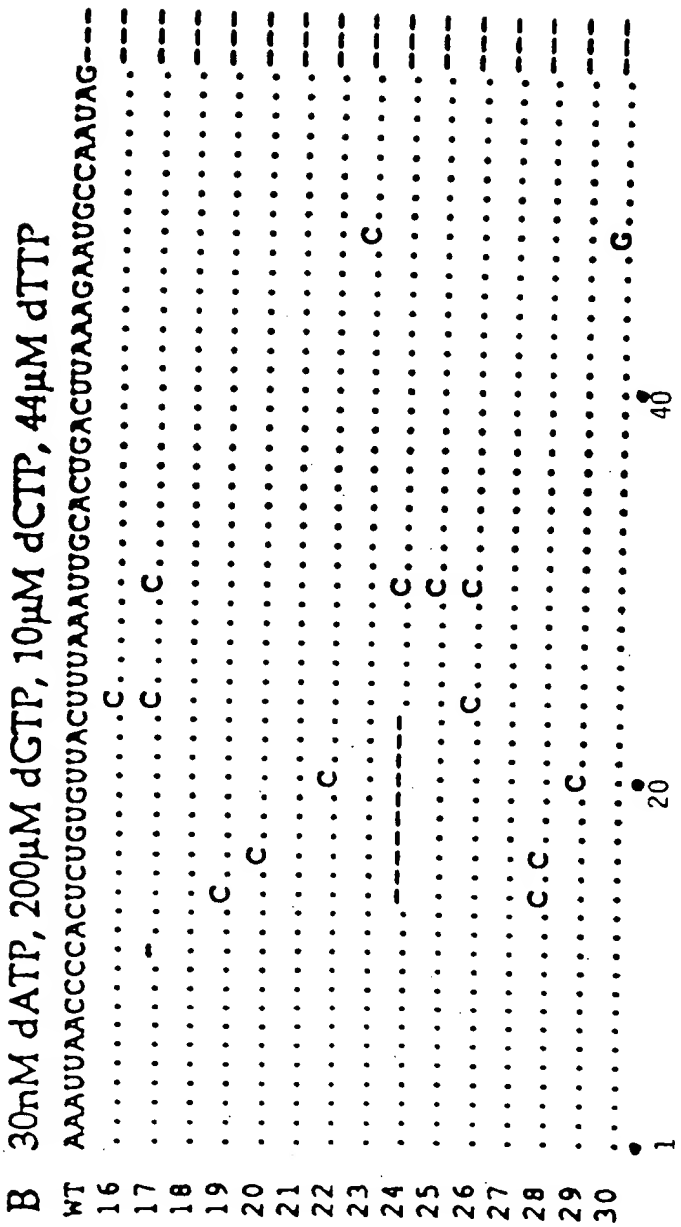


FIGURE 2 (Continued)

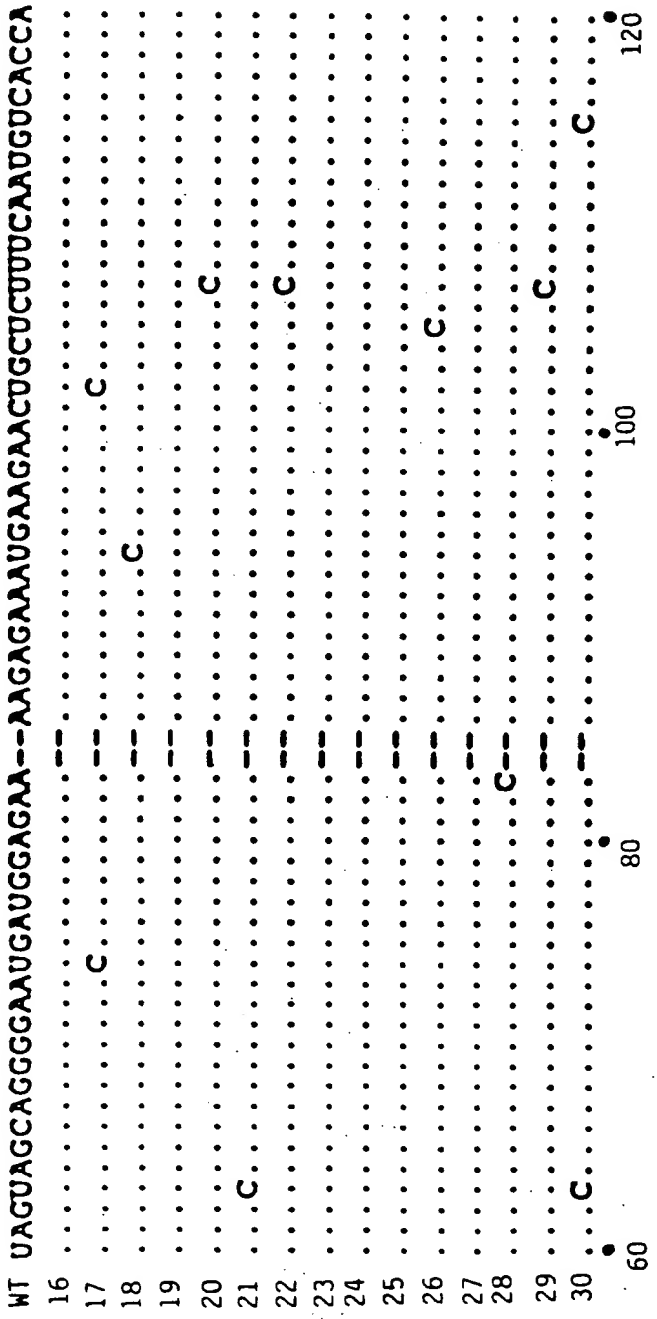


FIGURE 2 (Continued)

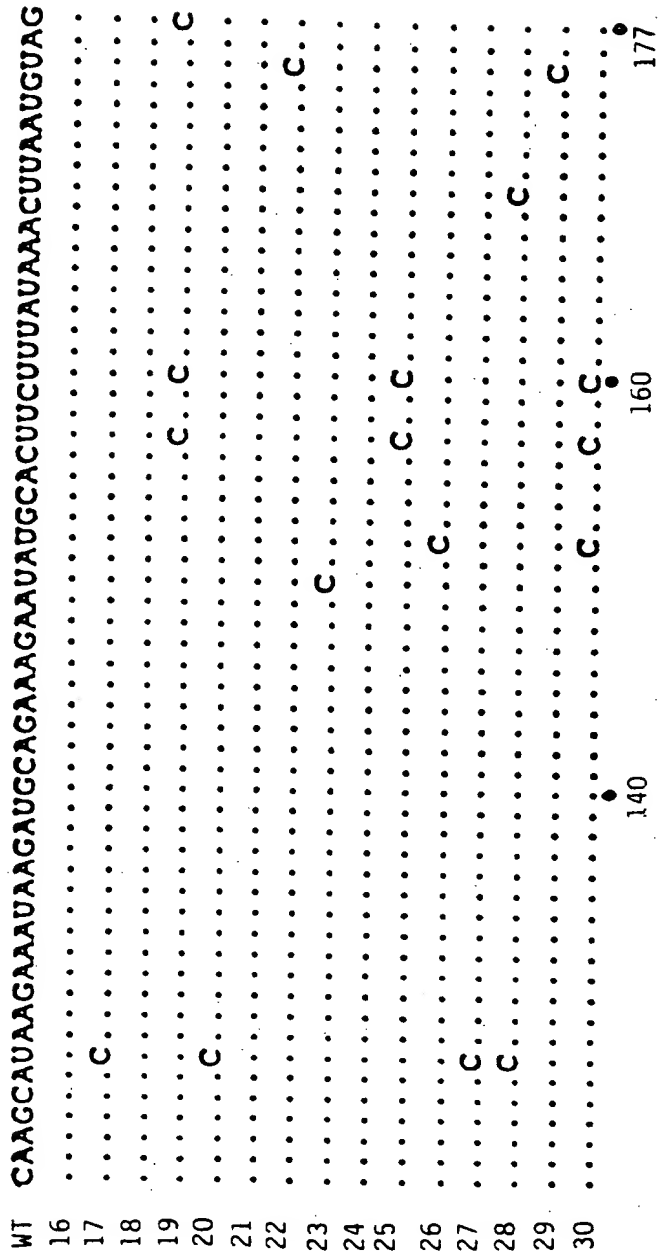


FIGURE 2 (Continued)

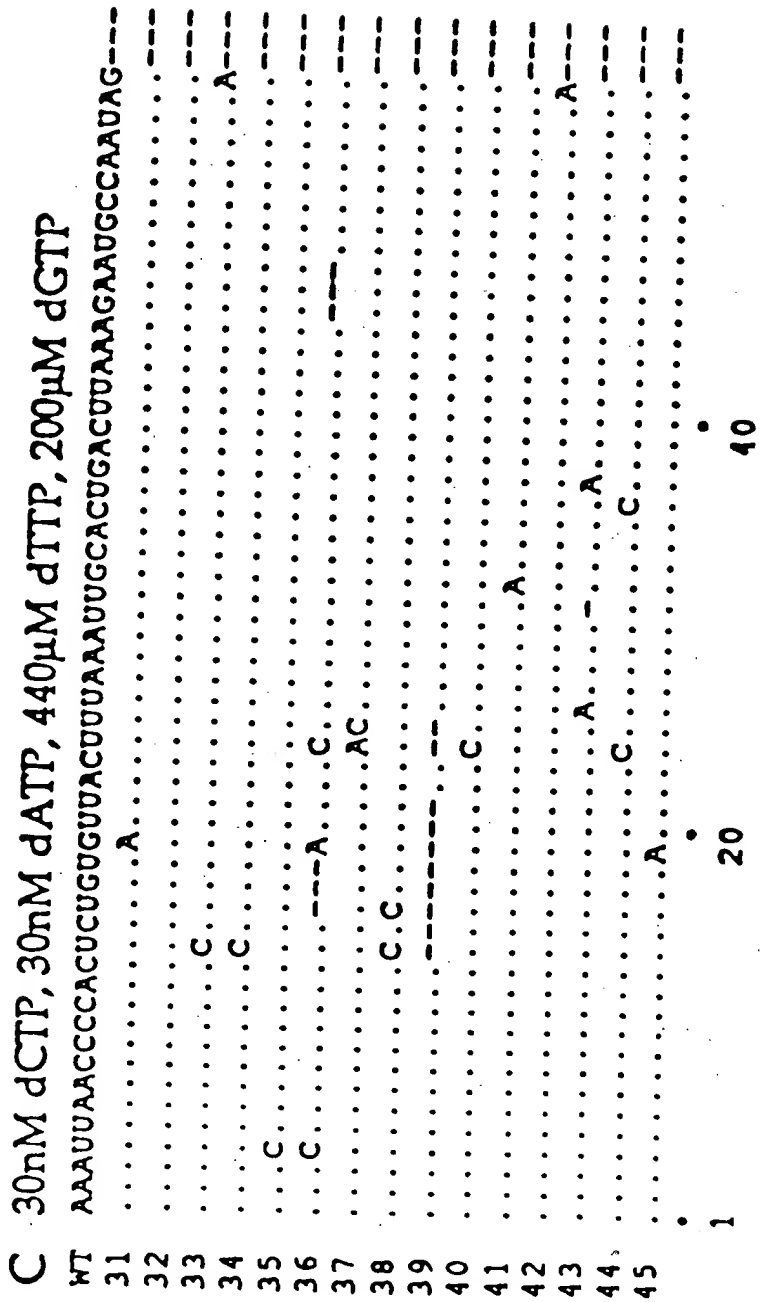


FIGURE 2 (Continued)

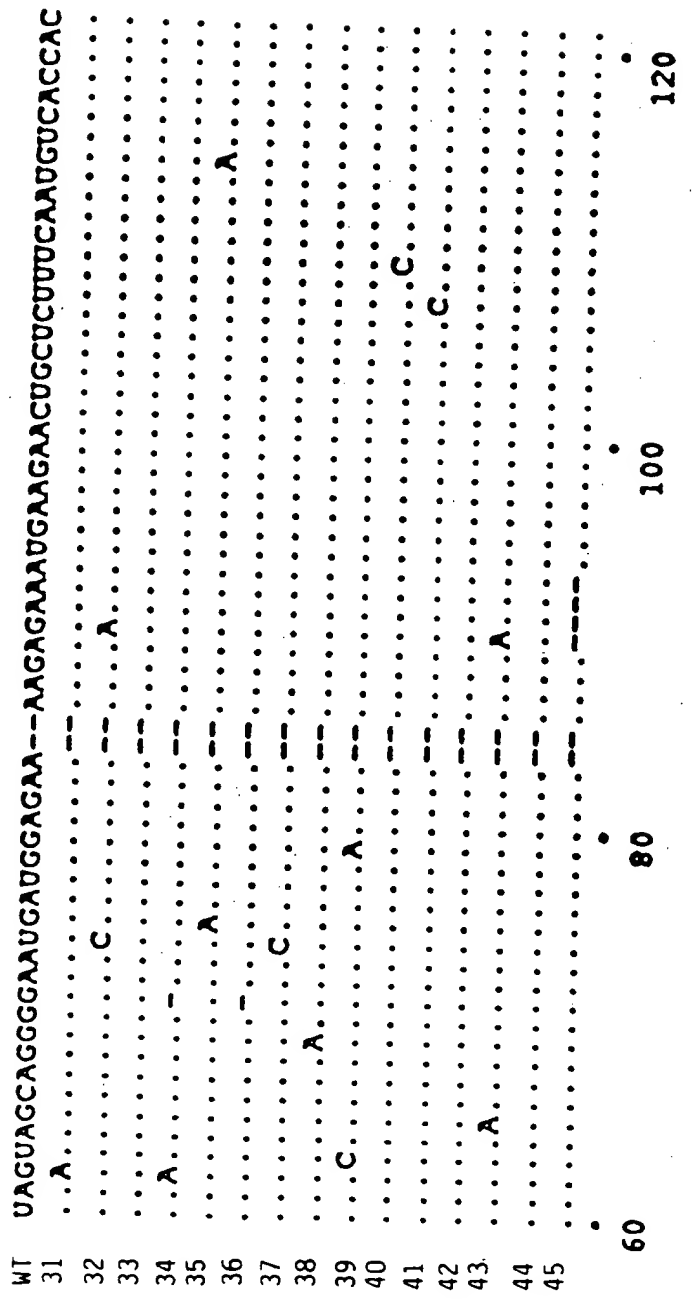


FIGURE 2 (Continued)

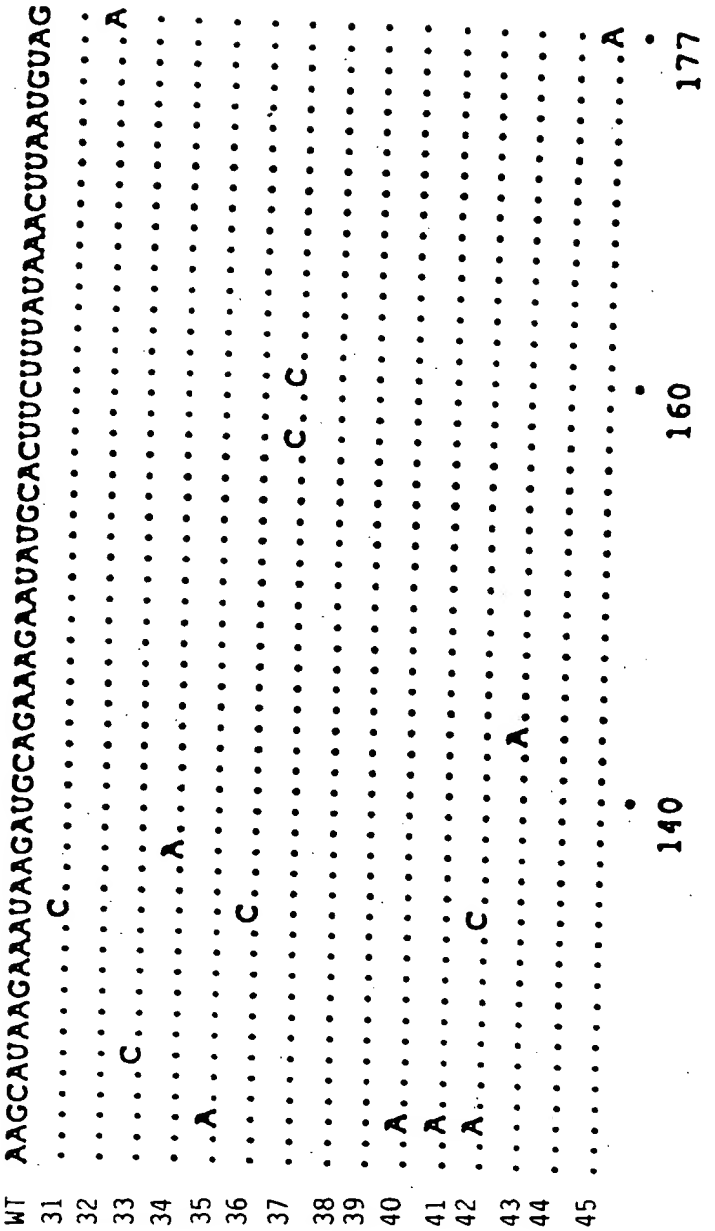


FIGURE 2 (Continued)

5' CGG GAGCTC CACAACAAAGGAACCAAATG forward primer

clamp SacI R67 specific

5' CCG GGATCC AACACCCAACCACCAACTTA backward primer

clamp BamHI R67 specific

FIGURE 3

forward primer

5' GCACGG GAGCTC ATTAACCCTCACTAAAGGGA CACAACAAAGGAACCAAATG

clamp SacI T3 promoter R67 specific

backward primer

5' GCACCG GGATCC AATTTAATACGACTCACTATAGGG AACACCCAACCACCAACTTA

clamp BamHI T7 promoter R67 specific

FIGURE 4

```
      SacI                    Met Glu Arg Ser Ser Asn Glu VAL Ser Asn Pro Val Ala Gly
5'  GAGCTC  CACAACAAACGAACCAA ATG GAA CGA AGT AGC AAT GAA GTC AGT AAT CCA GTT GCT GGC

    Asn Phe Val Phe Pro  Ser Asn Ala Thr Phe Val Met Gly Asp Arg Val Arg Lys Lys Ser
    AAT TTT GTA TTC CCA  TCG AAC GCC ACG TTT GGT ATG GGA GAT CGC GTG CGC AAG AAA TCC

    Gly Ala Ala Trp Gln  Gly Gln Ile Val Gly Trp Tyr Cys Thr Asn Leu Thr Pro Glu Gly
    GGC GCC GCC TGG CAA  GGT CAG ATT GTC GGG TGG TAC TGC ACA AAT TTG ACC CCC GAA GGC

    Tyr Ala Val Glu Ser  Glu Ala His Pro Gly Ser Val Gln Ile Tyr Pro Val Ala Ala Leu
    TAC GCC GTC GAG TCT  GAG GCT CAC CCA GGC TCA GTA CAG ATT TAT CCT GTT GCG GCG CTT

    Glu Arg Ile Asn Stop                                BamHI
    Gaa CGC ATC AAC TAA   TAAGTTGGTGGTTGGGTGTT    GGATCC
```

FIGURE 5

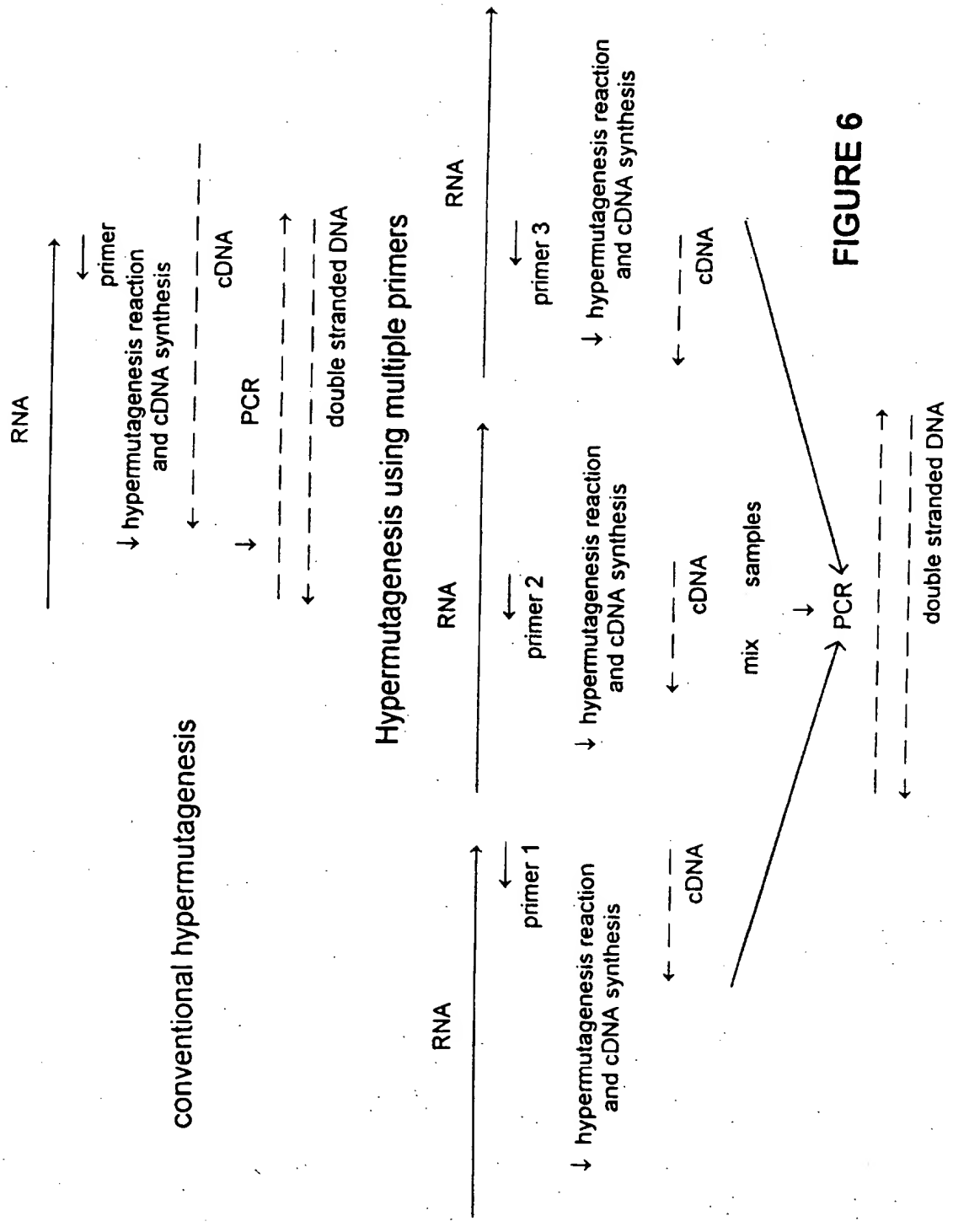
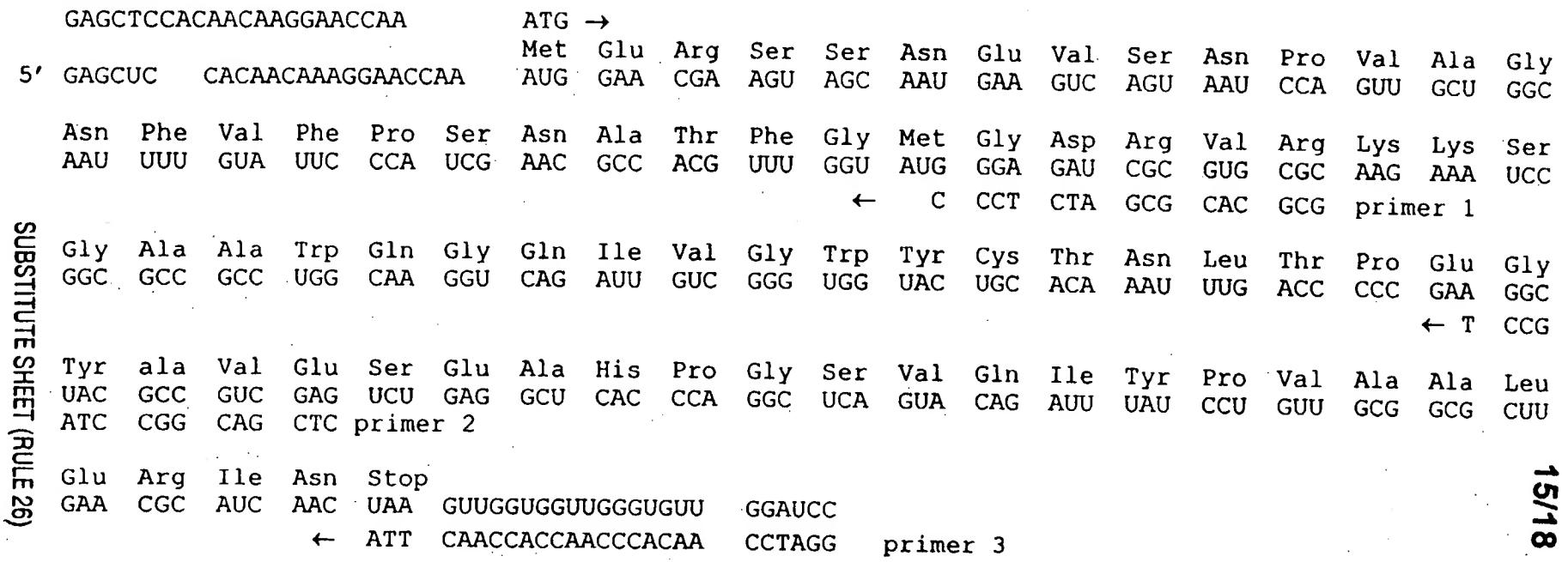

conventional hypermutagenesis
RNA
primer
hypermutagenesis reaction and cDNA synthesis
cDNA
PCR
double stranded DNA
Hypermutagenesis using multiple primers
RNA
primer 1
primer 2
primer 3
hypermutagenesis reaction and cDNA synthesis
cDNA
mix samples
PCR
double stranded DNA

FIGURE 6



Modified R67 gene sequence with respect to that given in Brisson and Hohn, Gene 28, 271-275, 1984
Amino acid sequence in the three letter code given above nucleic acid sequence

FIGURE 7

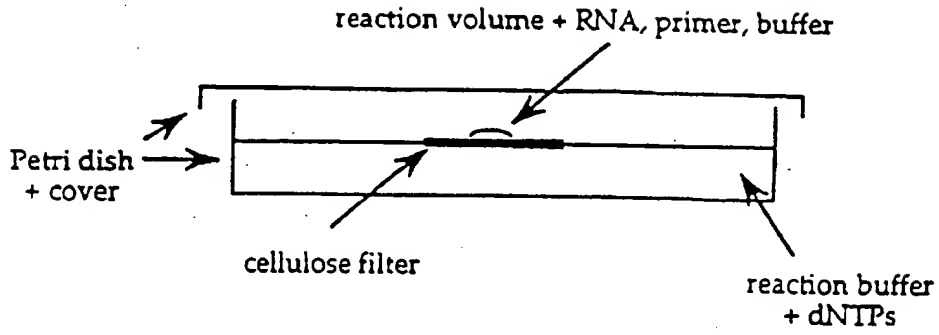

reaction volume + RNA, primer, buffer
Petri dish
+ cover
cellulose filter
reaction buffer
+ dNTPs

FIGURE 8

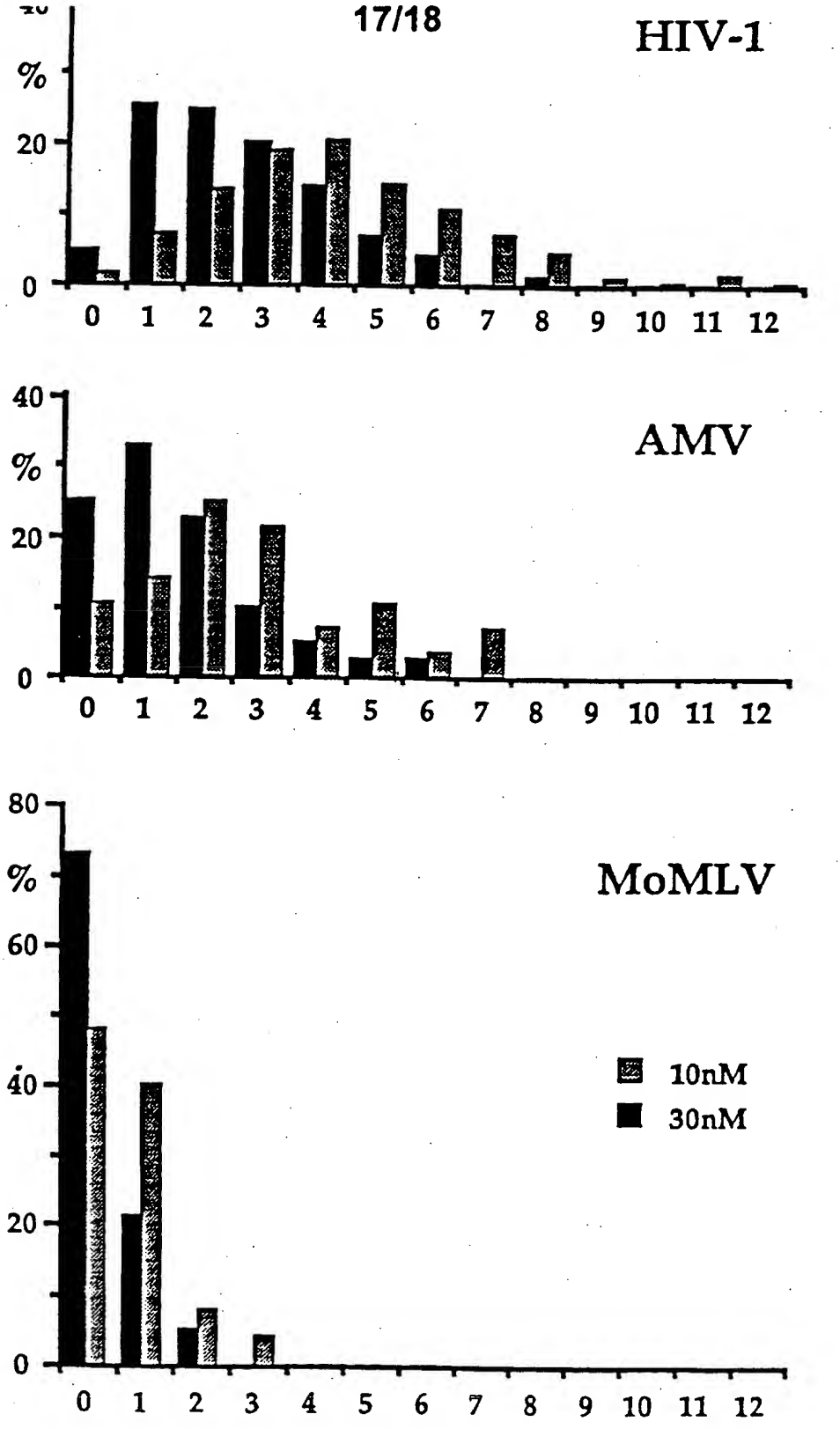

HIV-1
%
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
AMV
40
%
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
MoMLV
80
%
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
10nM
30nM
G→A transitions per clone

FIGURE 9

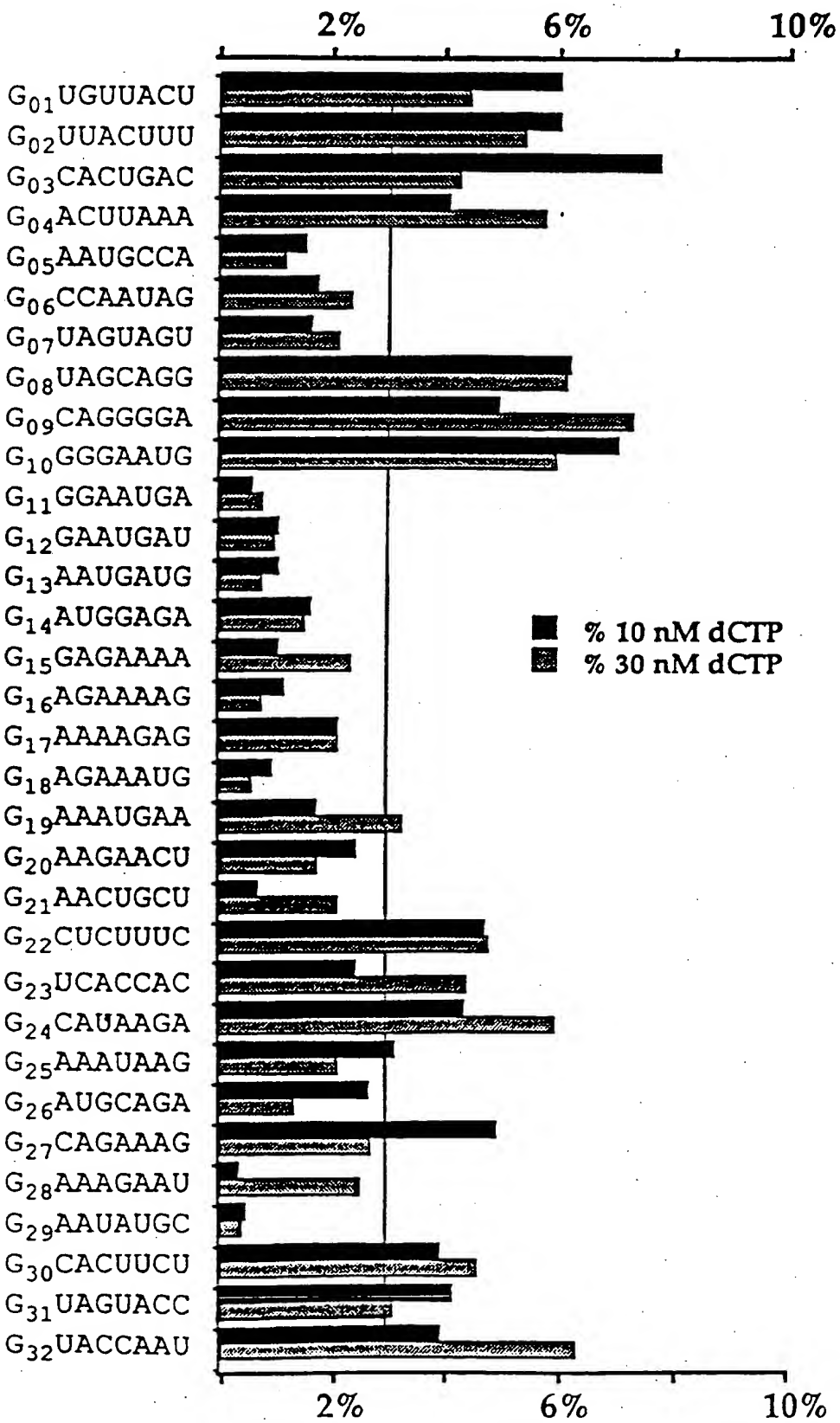

2%
6%
10%
G_{01}UGUUACU
G_{02}UUACUUU
G_{03}CACUGAC
G_{04}ACUUAAA
G_{05}AAUGCCA
G_{06}CCAAUAG
G_{07}UAGUAGU
G_{08}UAGCAGG
G_{09}CAGGGGA
G_{10}GGGAAUG
G_{11}GGAAUGA
G_{12}GAAUGAU
G_{13}AAUGAUG
G_{14}AUGGAGA
G_{15}GAGAAAA
G_{16}AGAAAAG
G_{17}AAAAGAG
G_{18}AGAAAUG
G_{19}AAAUGAA
G_{20}AAGAACU
G_{21}AACUGCU
G_{22}CUCUUUC
G_{23}UCACCAC
G_{24}CAUAAGA
G_{25}AAAUAAG
G_{26}AUGCAGA
G_{27}CAGAAAG
G_{28}AAAGAAU
G_{29}AAUAUGC
G_{30}CACUUCU
G_{31}UAGUACC
G_{32}UACCAAU
% 10 nM dCTP
% 30 nM dCTP
2%
6%
10%

FIGURE 10

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/EP 95/04749

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12N9/06 C12N9/12 C12Q1/68 C07K14/16
C07K16/10 G01N33/577 A61K38/00 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 285 123 (SUOMEN SOKERI OY) 5 October 1988 cited in the application	25,31-33
Y	see page 13, line 19 - line 39	1-24, 34-37, 39-41

X	PROTEIN ENGINEERING, vol. 2, no. 1, IRL PRESS LIMITED OXFORD, ENGLAND, pages 63-68, P.M. LEHTOVAARA ET AL. 'A new method for random mutagenesis of complete genes: enzymatic generation of mutant libraries in vitro'	25,31-33
Y	see page 67, right column, line 6 - page 68, left column, line 54	1-24, 34-37, 39-41

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1996	18.04.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROC. NATL.ACAD SCI., vol. 91, no. 8, 12 April 1994 NATL. ACAD SCI.,WASHINGTON,DC,US;, pages 3092-3096, J.-P. VARTANIAN ET AL. 'G to A hypermutation of the human immunodeficiency virus type 1 genome: Evidence for dCTP pool imbalance during reverse transcription' cited in the application see page 3092, right column, line 21 - page 3096, left column, line 2; table 1 ---	25,31-33
X	PROTEIN SCIENCE, vol. 2, no. 12, CAMBRIDGE UNIV. PRESS, ENGL., pages 2217-2225, P. PJURA ET AL. 'Development of an in vivo method to identify mutants of phage T4 lysozyme of enhanced thermostability' cited in the application	25,31-33
Y	see page 2218, right column, line 15 - page 2219, right column, line 3 ---	1-24, 34-37, 39-41
X	CELL, vol. 55, no. 2, 21 October 1988 CELL PRESS,CAMBRIDGE,MA,US;, pages 255-1265, R. CATTANEO ET AL. 'Biased hypermutation and other genetic changes in defective measles viruses in human brain infections' see page 255, right column, line 49 - page 262, left column, line 45 ---	32
X	WO,A,91 00353 (GIST-BROCADES N.V.) 10 January 1991	25,31-33
Y	see page 15, line 24 - page 16, line 21 see example 7 ---	1-24, 34-37, 39-41
Y	WO,A,92 18645 (DIAGEN INST MOLEKULARBIO) 29 October 1992 see page 5, line 14 - line 26; claims 1-4 ---	1-24, 34-37, 39-41
	-/--	

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 18, no. 4, 25 February 1990 IRL PRESS LIMITED,OXFORD,ENGLAND, pages 999-1005, S. KWOK ET AL. 'Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies' see page 1001, right column, line 12 - page 1005, left column, line 34 ---	1-24, 34-37, 39-41
Y	NUCLEIC ACIDS RESEARCH, vol. 17, no. 16, 25 August 1989 IRL PRESS LIMITED,OXFORD,ENGLAND, page 6749 R. SOMMER AND D. TAUTZ 'Minimal homology requirements for PCR primers' see page 6749, line 1 - line 24; table I ---	1-24, 34-37, 39-41
Y	GENE, vol. 82, no. 1, 15 October 1989 ELSEVIER SCIENCE PUBLISHERS,B.V.,AMSTERDAM,NL;, pages 83-87, G.F. JOYCE 'Amplification, mutation and selection of catalytic RNA' the whole document see figure 2 ---	1-24, 34-37, 39-41
Y	NATURE, vol. 370, 4 August 1994 MACMILLAN JOURNALS LTD., LONDON,UK, pages 389-391, W.P.C.STEMMER 'Rapid evolution of a protein in vitro by DNA shuffling' cited in the application see the whole document ---	37
A	TECHNIQUE, A JOURNAL OF METHODS IN CELL AND MOLECULAR BIOLOGY, vol. 1, no. 1, August 1989 SAUNDERS COMPANY,N.Y.,US, pages 11-15, D.W. LEUNG ET AL. 'A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction' see the whole document --- -/--	1-41

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/04749

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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